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Food quality control and authentication through coupling chemometrics to instrumental fingerprinting techniques

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CHAPTER 1

INTRODUCTION

1.1 QUALITY CONTROL OF FOOD

Food is any substance consumed to provide nutritional support for the body. It can be of plant or animal origin, and contains essential nutrients, such as carbohydrates, fats, proteins, vitamins, or minerals. The substance is ingested and assimilated by the organism in an effort to produce energy, maintain life or stimulate growth. Consequently, in recent years attention has been increasingly focused on what we eat, trying to ameliorate the quality of food consumed by improving the standard of living.

Today, most of the food energy consumed by the world population is supplied by the food industry so that, together with the food industry, the concepts of food safety and food quality were born at the same time.

Food safety is a discipline born to describe handling, preparation and storage of food in ways that prevent foodborne illness. These include a number of routines (rules) that should be followed to avoid potentially severe health hazards. The tracks within this line of thought are safety between industry and the market and then between the market and the consumer. Food safety includes the origins of food, the processes relating to food labeling, food hygiene, food additives and pesticide residues, as well as policies on biotechnology and food and guidelines for the management of governmental import and export inspection and certification systems for foods. In considering market to consumer practices, the usual thought is that food ought to

be safe in the market and the concern is safe delivery and preparation of the food for the consumer.

There are many agencies responsible for food safety monitoring. In particular, in the European Union (EU) the EU parliament is informed on food safety matters by the European Food Safety Authority (EFSA) created by European Regulation 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and enacting procedures in matters of food safety [1,2]. The EFSA provides scientific advice and effective communication regarding risks, existing and emerging, associated with the food chain.

Consumers worldwide always demand to have their foods of higher standards or better quality. However, the term "standard" or "quality" is often not unclear. "Food quality" may have different meaning. Food quality encompasses the quality characteristics of food that are acceptable to consumers. These include external factors as appearance (size, shape, color, gloss), texture, and flavor; internal factors such as chemical, physical and microbiological properties.

Food quality also deals with product traceability, e.g. of ingredient and packaging suppliers, should a recall of the product be required. It also deals with labeling issues to ensure there is correct ingredient and nutritional information.

There are many existing international quality institutes testing food products in order to indicate which are higher quality products. Founded in 1961 in Brussels, the international quality institute *Monde Selection* is the oldest one in evaluating food quality. During the analysis the products must meet the following selection criteria, required by the institute: sensory analysis, bacteriological and chemical analysis, the nutrition and health claims and the utilization notice. In short, the judgments are based on the following areas: taste, health, convenience, labeling, packaging, environmental friendliness and innovation. As many consumers rely on manufacturing and processing standards, the institute *Monde Selection* takes into account the European Food Law [3,4].

In order to guarantee food quality there must be an adequate quality control. The aim of quality control is to achieve a good and a consistent standard of quality in the product being produced as it is compatible with the market for which the product is designed, and the price at which it will sell.

Quality control is often considered under the following three headings:

Control of raw material

Control of the process

Control of the finished product

Each heading is important and indispensable. A given raw material may be examined and analyzed in different ways, with different techniques, depending on the information that we wish to obtain. In the same way, the finished products must be analyzed in order to check the chemical, physical, biological and/or organoleptic parameters. Clearly it is difficult to discuss raw material control without reference to process control. It is equally difficult to talk about process control without assuming that proper raw material control is carried out and that the materials are known to have reached the standard required for proper processing: they are simultaneously in operation. In planning a process control scheme, it is necessary first to list in sequence the steps in the process or to draw a flow diagram taking care to show the alternative processing steps, where these exist, to introduce changes in raw material. For each processing steps, one must identify the critical points, and define which trouble may arise which may be reflected in the quality of the finished product; for this reason it is necessary to establish controls in all these operations.

We often confuse "Quality Assurance" (Q.A.) with "Quality Control" (Q.C.). Quality control focuses on the product, while quality assurance focuses on the process. Quality control includes evaluating an activity, a product, process, or service while quality assurance aims to ensure processes are sufficient to meet clearly defined objectives. Further on, quality assurance ensures a product or service is created, implemented, or produced correctly, whereas quality control determines if the end product results are satisfactory or not.

Quality control in a typical food processing system begins right from the production stage of a food and runs till the stage of its sale and distribution. Some of the common quality control measures at each stage of a processed food are highlighted below:

- PRODUCTION
    - Control on the use of pesticides, veterinary drugs, and fertilizers.
    - Quality control at the time of harvesting.
    - Post-harvest handling particularly during storage (temperature, humidity and time control)
- PROCESSING
    - Use of Good Manufacturing Practices (GMPs)

- Application of Hazard Analysis of Critical Control Points (HACCP) approach to achieve optimum results with regard to the quality and safety of the product.
- The application of ISO 9000 series of standards to establish Q.C. regimes.

- DISTRIBUTION AND SALE
  - The ambient conditions under which food is stored or transported (e.g., time, temperature, humidity).

Developed countries have structured food safety regulatory systems that are increasingly comprehensive and more stringent. They are adopting a mix of regulatory approaches depending on the problem addressed, including process standards such as HACCP, performance standards for testing final products and even increasing labeling standards to communicate about food safety to consumers [5-7].

As above mentioned, the quality of end products is influenced primarily by the raw materials used. For this reason, close cooperation between agriculture and processing plants is needed. The farmers, in many cases, make agreements with the food industry, not only on the quantity of raw materials produced, but also all on their quality. In all cases, the raw materials must fulfill all standards requirements. Great attention is put on the presence of different kinds of contaminants such as toxic metabolites of microorganisms, toxic and heavy metals, residues of pesticides, the presence of undesired materials and others. In perishable raw materials, the chemical and microbiological quality of the raw material plays an important role and has to be controlled. For quality control of individual raw materials, different quality parameters are chosen, according to the quality requirements of the final products for which the raw material will be used [8]. Different evaluation methods based on different principles may be used. In particular, as far as the authentication of the quality of raw materials is concerned, usually rapid and accurate controls methods are preferred, for example through the use of NIRS (near-infrared spectroscopy) and HPLC (high performance liquid chromatography) but their choice depends on many factors [9-11].

Quality of finished food products is the most important indicator for the consumer. Finished products have to fulfill all requirements on quality. They should have the appropriate nutritive value, typical sensory characteristics and above all, meet all standards from a safety point of view. For this reason the quality control of finished products is the crucial point of the whole quality control chain. For the consumers, it is important that the quality of such products remains at the level declared by the producer during the whole storage period guaranteed. Labeling of food is also important; its purpose is to provide the consumer with the data

necessary for making an informed choice in the marketplace. The label must always bear the statement of identity; declaration of net contents; name and address of the manufacturer, packager, distributor; and a list of ingredients. The date of production and expiration date is most important, especially in perishable foods. National regulations usually require further information, such as nutrients and energy contents, and information about food additives with appropriate E number. The first step of quality control of finished food products starts in the factory. The producers are responsible for the quality of products. Therefore, they use the technological procedure in which the HACCP system is incorporated. This means that at least the critical control points are regularly examined. The high quality of produced foods is also important as a competition factor. In this respect, the producers are economically stimulated to produce foods of better quality than a competitive company. Factory laboratories are on high standard and are reasonably equipped. Moreover, when the analyses could not be possible without special and usually expensive equipment, the producers hire the services of special laboratories. The state protects consumers by running its own state control laboratories; their organization varies from state to state. Such laboratories, in developed countries, are well equipped, not only as far as the instrumentation is concerned but also with skilled and qualified analytical staff. Consumer organizations are also engaged in the food control system and play an important role. These organizations inform consumers about the results of quality comparative studies and draw attention to products that don't fulfill given quality requirements. Generally, the activity of such laboratories is focused on observation of the chemical composition, organoleptic properties, quality of packaging, microbiological state, presence of food additives and contaminants. Controlled products have to fulfill requirements for their given type of product and they especially have to be safe for the consumer. Such controls have to rule out the possibilities of health hazards and to guarantee that food products have not been adulterated. The food that the consumer receives from the farm or factory via food distribution system may exhibit important compositional changes that may be relevant to health or may not correspond to production claims, the label or trade agreements. The consumer is now more conscious about what he wants and the industry is eager to deliver the quality the consumer prefers. At the same time, scientific advances are making available tools and techniques that are more and more enhancing the sensibility, specificity and reproducibility of analytical methods. This information arising from the basic chemical sciences has assisted the analytical researcher in identifying new indicators of quality and authenticity of food. In many countries, mandatory provisions in food legislation are becoming more rigorous, especially for what concerns safety aspects. The objective of the

food analyst is to encompass, in addition to detection of adulteration, characterization of the food with respect to its source, the history of its handling, storage, preprocessing and so on [12-14].

The benefits of food laws to the consumers and the processing industry depend upon the effectiveness with which the laws are implemented. This requires not only a well-organized national infrastructure for inspection and quality control, but also the availability of reliable methods of analysis, which could be used to check the quality standards and safety. In this way, industries can be advised to make improvements in their food products and legal actions taken when necessary to protect the consumers. Therefore, in recent years, new methods for the analysis of food have been developed, together with the attempts to improve the existing ones. In this respect, one must recall that the analyses concern all aspects of a food, such as chemical, physical and microbiological. In this way, it is possible for instance to check that a food possesses certain nutritional parameters. In addition, it is possible to identify frauds, adulterations and guarantee to the consumers the quality standards of a food. Regarding the quality control of food, the key issues are both to check that a food has certain indices, determined according to well defined analyses, within specifications, and to identify the new parameters of control that are able to guarantee the quality of a specific food. In recent years, research has made significant progress in the knowledge of the main factors that contribute to define the quality of a food. Thanks to the development of new technologies, it has been possible to modify and improve the existing methods for the determination of the quality parameters and it has also been possible to create new methods for food analysis [15]. As mentioned previously, the development of fast and precise analytical methods are essential to ensure product quality, safety, authenticity and compliance with labeling.

1.2 TRACEABILITY AND AUTHENTICATION OF FOOD

Open markets and the development of the circulation of natural and processed foods in the European Union involves the necessity to inform consumers and predisposed organs about all the elements that contribute to the identification of food products.

Traceability means the ability to trace and follow a food, feed, food-producing animal or substance that will be used for consumption, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution. The need for traceability systems is well recognized throughout the world. In fact, traceability can protect consumers against deceptive marketing practices and/or frauds. Traceability can also allow to improve food safety, therefore it is a clear advantage for consumers and for food industry.

The possibility of tracing the origin of foodstuff is assuming an increasingly important role at the legislative level, as a tool that may allow to check whether quality requirements are met. It allows to establish the identity, history and origin of product. The evolution of the discipline of traceability is accomplished in two stages: in a first time, traceability was provided only for certain products (not-food) and for some individual foods; in a second step, it was extended to all products and foods. In the food industry, laws began to speak about traceability in relation to the organic production of agricultural products (Reg. CEE 24.06.1991 n 2092 art 9-12). The regulation disposed that Member States should ensure that the inspections relate to all stages of production, slaughter, cutting and any other preparation up to the sale to the consumer in order to guarantee, as far as technically possible, the traceability of products. Subsequently, on January 28th 2002, the European Parliament and the Council adopted Regulation (EC)178/2002 laying down the General Principles and requirements of Food Law. The aim of the General Food Law Regulation is to provide a framework to ensure a coherent approach in the development of food legislation. At the same time, it provides the general framework for those areas not covered by specific harmonized rules, where the functioning of the Internal Market is ensured by mutual recognition. It lays down definitions, principles and obligations covering all stages of food/feed production and distribution. According to this regulation, each business operator must be able to produce data about who their customers and suppliers are and have those systems and procedures to identify the product, so that it could be easier to withdraw it in case of danger for the consumers' health. However, it lacks a true commitment towards what has been called "traceability evolved", a wide range of methodologies aiming at the monitoring of various production processes, the control of mixing techniques and treatment of raw materials and the protection of the area of origin. Therefore, if on one side there are extremely positive national policy-making aimed at the preservation, protection and development of the "typical" local as a synonym for quality, on the other hand it is extremely complex, for the control authorities, to be able to provide those aspects of sanitation residing at the base of the rules on food safety for consumers. It is clear that, in a context so articulated, any action to market low-cost products derived from

imitation, adulteration and counterfeiting of traditional foods represents a potential risk to the health of consumers and it is also a damage for “legal” economies. Therefore, the development of innovative techniques and methods for the control of food products is a top priority in the development plans of both Community and National authorities, to pursue the objectives of increasing security and protection of the quality. The movement of food has no borders in a globalized supranational context. For these reasons the consumer world requires insistently a more detailed and accurate information about the nutritional parameters to guarantee the quality of food.

The authenticity of the product and its geographical traceability are therefore two fundamental aspects for a food.

The authentication of a food is the process by which it is possible to verify that the product conforms to the statements on the label, and possibly to what established in the in force regulations. In particular, the use of non-destructive, rapid, precise, accurate and highly performant analytical methods represents, for the authorities, a valuable and irreplaceable tool to verify the authenticity of a product. In addition, scientific innovation and technological evolution of instrumentation and methodologies, can allow to identify fraud and adulteration even if particularly sophisticated, or specifically designed to evade inspection of law currently applied. By definition, the authentication of a product invests issues that are very different among themselves, which largely depend on the type of fraud mainly practiced for each food. They include both the identification of possible adulteration and falsification, and the differentiation from other substitute products, the differentiation by age, or the identification of the geographical and varietal origin.

1.2.1 ANALYTICAL TECHNIQUES

There is no magic solution to improve the traceability but effective systems must comprise a number of key elements.

a) Regular labeling
b) Electronic labeling
c) Animal ear tags, passports

d) Production Records (one step forward-one step back)

However, these elements are not sufficient to ensure the traceability and authenticity of foods and the consumers are not completely protected against food frauds.
The analytical techniques, being *a posteriori* techniques, are essential for food safety, food quality control and for the traceability and authentication of food products. In fact, the analytical methods can provide feedback to prove that a system is working, troubleshoot and identify weaknesses and can provide traceability data where there is a breakdown in the chain. In addition the analytical techniques are effective internationally, and constitute a valid tool in order to prevent fraud and to confirm the authenticity of products. There are many emerging techniques available that can provide traceability information. Especially when used in combination, these techniques can provide extremely powerful tools.

The analytical techniques most commonly used for food authentication and traceability are the following:

• Stable isotope measurements (IRMS)
• Spectroscopic techniques (MIR, NIR, Raman, UV-VIS)
• Chromatographic techniques (GC, HPLC)
• Mass Spectrometric techniques (MS, MSMS)
• DNA-PCR methods
• Chemometric techniques (in next chapters the chemometric techniques used in this thesis will be discussed in detail)

Ratios of stable isotopes have been shown to be a valuable tool to discriminate foodstuffs according to their geographical origin and/or the technological processes applied during manufacture (production origin). In particular, determination of the isotopic ratios of the light elements, hydrogen ($\delta^{2}H$), carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), oxygen ($\delta^{18}O$), and sulfur ($\delta^{34}S$), the so-called bioelements, combined with ratios of heavy isotopes ($\delta^{87}Sr$) and trace elements have been used successfully to provide information on the origin of food products [16-20]. However, although some official methods using isotope ratios have been introduced, they are usually reliant on commodity specific databases which are expensive to produce and to maintain.

Spectroscopy is the study of interaction between photons of radiation and molecules. Among the most widely used spectroscopic techniques for the authenticity of the food, there are MIR (mid-infrared) and NIR(near-infrared) spectroscopy. Even if the electromagnetic radiation used is of different frequency (range 4000-400 cm^{-1} is referred to as mid-infrared and 12500 and 4000 cm^{-1} is known as near-infrared radiation), the response of the instrument consists of absorption bands due to chemical compounds, that can be observed, in the spectral regions of the MIR and NIR, as a result of molecular vibrations of these compounds thus giving rise to spectral signatures which are characteristic of the food composition and which may be considered as "fingerprints" of the food [21,22].

Chromatographic methods are widely used for the measurement of the 'fingerprints' of foodstuffs. Gas chromatography (GC) and high performance liquid chromatography (HPLC) provide high-resolution compound separations, and can be used in conjunction with different detectors such as a diode array detector (DAD) or a mass spectrometer (GC-MS, GC-MS/MS, LC-MS, LC-MS/MS). The mass spectrometers are highly sensitive and universal, able to detect almost any organic compound, regardless of its class or structure. As reported for spectroscopic profiles, the chromatographic profiles may be used as the fingerprint of the food to control the quality of food and to guarantee its authenticity [23,24].

Analysis of specific nucleic acids in food allows control laboratories to determine the presence or absence of certain ingredients in complex products or the identification of specific characteristics of single food components. In food analysis, DNA detection is increasingly applied as an answer to different needs, such as for GMO detection, microbial pathogen determination, assessment of the presence of undeclared allergenic ingredients [25]. These analyses are based on nucleic acids probes, including the polymerase chain reaction (PCR), which allow the detection of minute amounts of degraded nucleic acids and their sequence. These methods may be also used for the identification of meat or fish species and the recognition of genetically altered foods [26].

1.3 REVISION OF METHODS OF FOOD CHEMICAL ANALYSIS

As mentioned in the previous paragraphs, the control of food quality, food safety, traceability and authentication of food have considerable importance. Therefore, scientific research is increasingly addressing the development of new methods that can ensure the geographical/botanical traceability [27]. In particular, in recent years, some successful examples of application of fingerprinting techniques for assessing the origin of foods have been reported in the literature [28,29]. In this context, the possibility of relying on the outcome of a fingerprinting technique to authenticate the origin of a foodstuff has a high potential as it would allow the traceability of the product without being tied to the labeling or production records.

Parallel to this – and always with the aim of guaranteeing the consumers by assessing the quality of a food, especially if with added value, and to characterize foods identifying the nutraceutical components – scientific research has also put a big effort in the revision of the traditional methods of food chemical analysis, with the objective of developing methods with better performance compared to the ones currently used for the determination of the constituents of foods. In fact, even if several methods for the chemical analysis of some characteristics of the food already exist, for instance all the analytical methods described in the laws, the continuous innovation and technological development have made researchers trying to develop methods for food analysis resulting in better performance than the currently adopted ones in terms of accuracy and precision, trueness, limits of detection and quantification etc. Together with these aspect, also the possibility of reducing the times and costs of analysis without loss in accuracy is also often investigated, as it could allow carry out a higher number of controls in the same timespan and with the same budget.

On the other hand, in recent years the international community is laying attention on environmental issues and on green chemistry. Green chemistry is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances [30]. In addition to being innovative, the approach of green chemistry is, at the same time, not-regulatory and attentive to the economic aspects. Therefore, developing methods for chemical analysis with the intention of preventing the pollution can be defined a new scientific approach to eliminate or minimize the environmental problems. More generally, whenever possible, it is appropriate to replace the traditional obsolete test methods

with others who maintain their functional efficacy while reducing toxicity to humans and the environment.

In this context, in 1999, the concept of green analytical chemistry was introduced, together with some representative examples [31]. In 2001, Namiesnik suggested that the twelve principles of green chemistry could be used to formulate the "green" character of Analytical chemistry and identified four priorities [32]:

1) Elimination (or, at least, a significant reduction) in the consumption of reagents, in particular organic solvents, by the procedures of analysis;
2) Reducing emissions of gases and vapors, as well as liquid and solid waste generated in the laboratories of analysis;
3) Elimination of reagents that show high toxicity and / or ecotoxicity from analytical procedures (e.g., by replacing the benzene with other solvents);
4) Reduction of energy consumption of the work required by the procedures of analysis;

In accordance with the guidelines of green chemistry and the priorities identified by Namiesnik, the following seven principles were suggested for green analytical chemistry:

1) Not polluting analytical techniques and production processes
2) Efficiency in terms of time, labor and energy consumption;
3) No or minimal sample preparation;
4) No or minimal destruction of the sample;
5) Low or even zero cost in terms of reagents and release of waste, and elimination of highly toxic reagents from analytical procedures;
6) Analysis in situ/in vivo or real-time process monitoring;
7) Simple/portable instrumentation while maintaining high selectivity and sensitivity;

It is necessary to point out how these seven aspects must be considered as a whole rather than individually when it comes to green analytical chemistry. It is evident, in fact, that their complete application is related to an ideal condition as it is practically impossible that all analytical methodologies can satisfy all these aspects: the important thing is to make an analytical procedure as green as possible.

In the traditional analytical chemistry approach, an analytical procedure is presented as a series of subsequent steps: sampling (go to the selected site, collecting samples for analysis, transport to the laboratory and possible pre-treatment to maintain sample integrity), sample preparation (eg, dissolution, digestion, separation, enrichment, etc.. all processes "cost "in terms of time and energy); measure (transformation of the analytes in" measurable form "with

procedures which may require energy, reagents, and can lead to release of polluting products); waste disposal (residues of the sample, reagents used, products reaction, etc.).
Therefore, the conventional procedures of chemical analysis, often necessarily destructive, are generally expensive not only because they consume time, reagents and energy, but also because they produce waste that, being dangerous to humans and to the environment, require special treatments for disposal. The aim of green analytical chemistry is to follow analytical procedures that generate less hazardous wastes and which are more secure to use both for both man and environment [33].

1.4 THE ROLE OF CHEMOMETRICS IN FOOD ANALYSIS

Chemometrics, according to the definition of the International Chemometrics Society, is "the chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments, and to provide maximum chemical information by analyzing chemical data". Already from the definition, the importance of chemometrics for the chemist is clear. Chemometrics has a key role in all areas of chemistry, including analytical chemistry. Consequently, chemometrics is a necessary and powerful tool in the field of food analysis and control [34]. It is widely known that the application of advanced statistical and mathematical methods has been continuously increasing in food science, once the use of such techniques has allowed the extraction and identification of important results from complex data matrices. Nowadays these statistical techniques are necessary for the academy and food industry during the development and evaluation of food products and processes, as well as during the study of the mechanisms underlying different phenomena that may affect the product's quality or unit operations in the food development. Thus, the interest and application of new and complex statistical and mathematical techniques in food science has significantly increased [35,36].
The issues related to authentication, typicality, traceability and overall quality of foods are of particular importance for researchers, regulatory entities and most importantly for consumers. The need to guarantee quality (nutritional value, absence of adulterations, traceability, food safety, typicality, sensory properties including image analysis and other intrinsic quality parameters) has led researchers and sanitary vigilance authorities to develop and use effective

statistical tools to investigate food-related problems and to address limitations on processes and shelf life. Once food matrices become complex, the way to investigate and try to solve problems related to sensory, chemical, physical and rheological issues is multivariate and thus require multidimensional data. Thus, the use of multivariate statistical techniques has gained strength in Food Science, especially for monitoring the unit operations and the quality of food products, including beverages.

Technological innovation implies the use of increasingly sophisticated instruments, through which it is possible to face and overcome analytical problems otherwise unsolvable. The chemist has at its disposal tools more precise, accurate, sensitive and which allow to determine qualitatively and quantitatively compounds even in trace. These techniques and tools also result in thousands of data in which useful information is often "hidden". Often we have too much data and too less information. In fact, a serious imbalance is developing in science, between the technical capacity to generate lots of good data and the human capacity to interpret and understand all these data. Indeed, it should be emphasized that the fact of having many data is not a synonym of having many information, in fact data is not the same as information. The fact that the analytical chemist has innovative tools available, almost always very expensive, but from which he then fails to obtain all possible information without fully interpreting them is, as once Harald Martens, a famous norwegian chemometrician, said, “like having a grand pianos and playing with only one finger”.

Near-infrared spectroscopy represents one example. The information enshrined in an entire NIR spectrum is poorly selective, as it depends on a particularly large number of physical variables, chemical and structural properties, which often make the recognition of differences between the samples subjected to analysis very difficult. To obtain useful information, as for instance the amount of a particular substance in a food sample, or the identification of possible differences between samples subjected to NIR analysis, it is necessary to use mathematical and statistical techniques without which it would be impossible to solve some analytical problems.

Chemical analysis of food is also part of the issue of traceability and fingerprinting techniques as a tool to characterize, identify, and ensure the authenticity of the food. In fact, the term “fingerprinting techniques” describes a variety of analytical methods that can measure the composition of foodstuffs in a non-selective way such as by collecting a spectrum or a chromatogram. Mathematical processing of the information contained in such fingerprints may permit the characterization of foodstuffs. Fingerprinting techniques produce a large volume of information. Most of the information may not be useful for solving the problem of

authentication or identity confirmation. Mathematical tools, such as classification models, must be applied to these signals to extract that information which is helpful to solve the problem being investigated [37]. Simply, a model is a mathematical equation which can convert measurements, may be many hundred or more, made by one or more fingerprinting techniques into indicators or numbers that are easily interpretable; when mathematical and statistical methods are applied to the fingerprint of a given sample, the outcomes of the corresponding model can for example represent the answer to the question "Is this food what it claims to be on the product label?"

Without these mathematical processes, it would be impossible to carry out the classification of foods, especially if there are thousands of variables such as the points that constitute a spectrum, a chromatogram or the innumerable chemical compounds that describe and characterize a food.

The mathematical and statistical techniques play a key role also in the context of Identity Confirmation (IC). Methodology to confirm that a food is in compliance with claimed identity. An important aspect of food production is to produce a good which always has the same characteristics and therefore, by extension, with the same fingerprinting. The food industry can verify the consistency of their product using fingerprinting techniques and mathematical techniques [38].

Other issues that can be addressed with chemometrics concern process monitoring and the quality control of foods. In fact, to ensure the control of the quality of a food, which depends on several factors/variables, a multivariate analysis of the entire system is then required. Indeed, it is not sufficient to carry out quality control or monitoring of a production process in a univariate mode, because the system is a multivariate system. Therefore, there is an increasing need for the analytical chemist to use mathematical tools which allow to treat systems, more or less complex, also described by thousands of variables. Accordingly, in quality control in general, and in particular in food quality control, there has been a transition from using systems such as the univariate control charts to multivariate systems [39].

When dealing with n quality variables, the usual approach consists in verifying whether the value of each variable measured on the final product is inside some predefined limits. If all the variables are inside the range, then the product is said to be within specification. Probably this statement is not always correct. The problem with using univariate control charts for separately monitoring key variables on the final product is that the variables are not independent on one another, and none of them adequately defines product quality by itself.

Product quality is defined by the simultaneous correct values of all the measured properties; thus, a multivariate property requires multivariate analysis methods [40].

Chemometric plays an important role also in the choice of the experiments to be carried out for the optimization of an analytical method, allowing for the development phase of an analytical method a saving of time and money. In fact, the use of experimental designs makes it possible to define *a priori* the experiments to be executed and the data to be collected. While the standard way of developing an analytical method is very often to select possible influencing factors, vary them one-by-one and evaluate their influence on the response(s) of interest (OVAT – One Variable at A Time – approach), experimental design represents a valid alternative to this approach. In fact, it is an even better alternative because for a given number of experiments the experimental domain is more completely covered and interaction effects between factors can be evaluated.

Mention was also made about the development of the analytical instruments of analysis that enabled to overcome analytical problems, but there are issues that can be overcome by the application of chemometric methods. Unstable baselines occur in many types of instrumental measurements. They can cause severe problems, especially when detection limits are approached [41]. These baselines hamper the interpretation of spectra or chromatograms. In addition, the baseline varies greatly from spectrum to spectrum (or from chromatogram to chromatogram), even for similar samples. In quantitative analysis, these inconsistent baselines are able to reduce the simplicity and robustness of a calibration model that is built on these spectra or chromatograms. In these cases the application of mathematical processing tool can help to improve the baseline allowing a better interpretation of the data.

Chemometric comes to the aid of the analytical chemist also to solve problems related to the shift of the retention times which may be due to multiple causes such as variations in temperature between a chromatographic run and another run, the chromatographic column not being well conditioned, etc [42]. In fact, the importance of always having the same retention time for the same analyte present in different samples is rather obvious, especially when analyzing complex matrices such as foods. The "shift" is not, however, a phenomenon concerning only the retention time in chromatography. Many analytical techniques yield data where the same underlying factor may result in signals at different positions or which may have different ‘durations’ depending on the specific analytical conditions.

1.5 AIM OF THESIS

Food safety and authenticity are, nowadays, themes of growing interest and increasing importance. As a result, the European Union has issued over the years, regulations to guarantee consumers relating to food safety and traceability [43,44] and, together with the monitoring bodies, encourages the development of effective methods to combat food fraud not only caused by the fraudulent addition of substances, but also those due to misrepresentation on the label [45].

In addition to developing new methods for the analysis of foods that make it possible to check the authenticity of a food and to discover new food fraud, research is moving towards the improvement of the performance of the existing ones, even with the support of mathematical-statistical methods and therefore with chemometrics.

For these reasons, the aim of this thesis was to develop new methods of chemical analysis for the verification of the authenticity and the traceability of food. In this context, the developed methods focus on the verification of two aspects which are closely related:

i) the chemical characterization of foods, in terms of monitoring their composition and quantifying their constituents
ii) the identification of the origin of foods

On one hand, therefore, chemical methods of analysis for the determination of some components presents in different foods have been developed and validated.

In particular, a spectroscopic method based on NIR spectroscopy for the determination of the some of the indices required by law for the quality control of honey samples – water, reducing sugars and hydroxy methyl furfural (HMF) – has been developed. Another purpose was to develop an innovative method based on the extraction with microwaves and subsequent chromatographic analysis for the determination of the quality of saffron.

Concurrent acetylation-dispersive liquid-liquid microextraction (DLLME) combined with gas chromatography mass spectrometry (GC-MS) has been proposed, for the first time, for the sensitive determination of several polar benzotriazolic compounds in water samples. In fact, even if the water is not considered a food, the ingestion of water in some form is widely recognized as essential for human life.

The methods of analysis have been improved compared to traditional and law methods, by reducing the economic costs and times of analysis and also considering the environmental

impact, trying to reduce the environmental costs by eliminating or minimizing the use of toxic and hazardous solvents.

On the other hand, chemical methods have been developed to verify and authenticate the origin of foods. Specifically, a method for the analysis of extra virgin olive oil, which allows to identify and discriminate Sabina PDO extra virgin olive oils from the others, was developed and validated.

Analogously, the same approach was followed to verify the origin of two other high value-added food products, honey and saffron. In particular, a method of analysis that allows to determine both the geographical (Italian/non-Italian) and the botanical origin of different honeys, was designed, developed, optimized and validated. The same strategy was followed to design and optimize a method for characterizing the geographical origin of saffron, also taking into account the possible differences in the growing and production processes.

Given the different foods and the different problems faced, the research was articulated and configured in a way which has necessarily involved the use of multiple methods of analysis. Indeed, depending on the type of food and the issues to be solved, the most appropriate and cost-effective strategy, both in terms of analytical platform and of chemometric techniques chosen, was always selected.

More in detail, the experimental work was focused on the following research topics:

1- **Olive oil**: Geographical traceability of extra virgin olive oils from Sabina PDO by chromatographic fingerprinting of the phenolic fraction coupled to chemometrics (**chapter 3**)
2- **Honey**: Geographical and botanical traceability of honey by chromatographic and spectroscopic fingerprinting coupled to chemometrics (**chapter 4**); Determination of quality parameters of honey by Near-Infrared spectroscopy and chemometrics (**chapter 5**)
3- **Saffron**: Determination of quality of saffron samples by microwave-assisted extraction and chromatography (**chapter 6**)
4- **Water**: Determination of benzotriazoles in water samples by concurrent derivatization-dispersive liquid-liquid microextraction followed by gas chromatography mass spectrometry (**chapter 7**)

CHAPTHER 2

CHEMOMETRIC METHODS

2.1 EXPERIMENTAL DESIGN

In analytical chemistry, especially in method development, it is of utmost importance to be able to optimize all parameters that can affect the performances of the method itself. In this framework, the objective is to perform a limited number of experiments – ideally as few as possible, but at the same time to be able to determine how the experimental variables influence the outcomes of the analysis and whether there are any interactions between the factors.

Based on these assumptions, it is evident how in all cases where there is the need to optimize a process or a response, as for instance an extraction procedure, or the yield of a reaction, or when it is necessary to evaluate the incidence of multiple factors (experimental variables) on a procedure, it is advantageous and often essential to think and operate in a multivariate way. Indeed, varying one variable at a time while keeping all other constants, the so-called OVAT approach, apart from requiring in general a significantly higher number of experiments to be performed, almost always lead to suboptimal solution, as it doesn't take into account the possibility that factors interact with one another.

An experimental design can be considered as a series of experiments that, in general, are defined a priori and allow the influence of a predefined number of factors (experimental variables) in a predefined number of experiments to be evaluated [46].

In order to properly design the experiments to be conducted, the first step is to define the analytical problem (what do we need to investigate?), what are the experimental variables that screened and controlled and what is the response(s) that better describe the propertie(s) to be optimized? Once the experimental variables and the responses have been clearly defined, the experiments can be planned and performed in such a way that a maximum of information is gained from a minimum of experiments.

At each of the design points, one or more responses are determined, so that the effect of the controlled factors and their interactions on them can be evaluated. For instance, in the simplest case when a factor is controlled only at two levels, then its effect can be calculated as the difference between the average value of the response obtained when this factor is at its high and at its low levels. The relevance of the effects (i.e., the significance of their difference from the variability which can be ascribed to the experimental error) is either statistically or graphically evaluated [47].

Different types of experimental designs are available to the analytical chemist, depending on the analytical problems to face, and, in particular, depending on the number and type of variables that one wants to optimize. In this framework, the different kinds of experimental designs can be roughly divided in two categories, those aimed at screening and the ones for optimization [48].

Screening designs are used to search for possibly important factors during method optimization or in robustness testing. They can be used if there is little knowledge of the possible factors that may affect the response: in these cases, all the possible factors that can influence the results of a method should be selected. With the use screening designs, it is possible to identify the factors that have a major influence on the response(s) of interest. Generally, two-level designs are used for screening, as they allow screening a relatively high number of factors in a rather low number of experiments. These designs can also be used to verify the robustness of an analytical method. In this context, the difference between the screening and robustness testing lies in the amplitude of the explored experimental domain, i.e. in the interval between the two levels of the factors [49]. Indeed, for any given factor, a relatively large interval is considered for optimization, while in robustness testing the intervals are much smaller and do not exceed much the experimental error.

The optimization of a method can be performed with a stepwise strategy. This means that groups of experiments can be performed sequentially. For instance, it is possible to make a first experimental design in a given experimental domain and, depending on the result, repeating another experimental design but choosing a different range of variability for the

factors to be investigated. This process can be repeated step by step until a pre-determined criterion is met. For example, if for the optimization of an extraction method a recovery of 80% is sufficient and, with the experimental design, it is possible to identify the portion of the experimental domain that allows an extraction efficiency higher than 80%, it is not necessary to perform additional experiments. If, instead, the best experimental setting still does not result in a recovery of at least 80%, then there is the need to perform additional experiments by extending the experimental domain in the direction of the optimal conditions obtained with the previous experimental design.

HOW TO START

The first step of any experimental design consists in determining which factors could influence the response(s) and in choosing the domain of variability for each controlled factor. Sometimes one knows which factors have an effect on the response, but often this information is not available. In this case, it is possible to start writing down all the possible factors that could have an effect on the response and make a screening of which factors may have an effect by using the highest possible fraction of a factorial design or the corresponding Plackett-Burman‘s designs which are performed on two levels with a number of experiments increasing by multiples of 4 [50]. After choosing the factors, it is necessary to fix the limits of the experimental domain, i.e. the extreme levels for each experimental variable. The next step is often to obtain a model that describes in a quantitative manner the effect of the factors on the response. Finally, based on the model, one tries to find the optimal conditions, or, in other words, the values of the factors that result in the best features of the product, process or procedure studied [51].

EXPERIMENTAL MODELS

The response Y of an experiment (the area of a peak, the intensity of a signal, etc.) is influenced by the experimental conditions. Mathematically $Y = f(x)$. The function $f(x)$ is a polynomial function that, within the experimental domain, relates the controlled factors to the response. There are three types of polynomial models that describe the Y response. The first and simplest is the linear model, where the relationship between the experimental variables and the response is linear. For instance, in the case where two factors x1 and x2 are controlled:

$$y = b_0 + b_1 x_1 + b_2 x_2 + e \qquad (1)$$

e being the residual, i.e. the portion of the variability in the response y not explained by the model.

On the other hand, if there are interactions among variables, terms accounting for these interactions should be added. Usually, only second order interactions, i.e. those involving pair of factors, are considered to be possibly significant. Under this assumption, in the case of two factors, equation 1 transforms to:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 + e \qquad (2)$$

These two models, linear model and second order interaction model, are the ones most often used to do a screening and/or robustness tests.

In all the cases where it is not possible to assume a linear relationship between the experimental variables and the response, higher order polynomial terms should also be included. However, the models customarily used in experimental design very rarely exceed second order polynomials, meaning that a quadratic function is fitted to the data. In the case of two controlled factors, this translates to:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + e \qquad (3)$$

Of course, even though the functions reported in equations 1-3 refer to the case when only two factors are controlled, they can be easily generalized to a higher number of variables.

The polynomial functions described contain unknown parameters (b0, b1, b2, etc.), which need to be estimated based on the results of the experiments carried out and for each model an appropriate experimental design exists.

FULL FACTORIAL DESIGN

The full factorial design with two levels are used to determine if some factors and / or interactions between two or more factors have effect on the response, and to estimate the magnitude of this effect. It requires that experiments be conducted at all possible combinations of the two levels of the k factors studied. Therefore, the number of these experiments is 2k, which is also the way these designs are indicated [52].

As an example, the experimental matrices describing the factor levels for the full factorial designs in the case of 2, 3, and 4 controlled factors are reported in Tables 2.1-2..3.

Table 2.1: full factorial design for 2 factors

experiment number	variable 1 b(1)	variable 2 (b2)
1	-1	-1
2	-1	+1
3	+1	-1
4	+1	+1

Table 2.2: full factorial design for 3 factors

experiment number	variable 1 (b1)	variable 2 (b2)	variable 3 (b3)
1	-1	-1	-1
2	-1	-1	+1
3	-1	+1	-1
4	-1	+1	+1
5	+1	-1	-1
6	+1	-1	+1
7	+1	+1	-1
8	+1	+1	+1

Table 2.3: full factorial design for 4 factors

experiment number	variable 1 (b1)	variable 2 (b2)	variable 3 (b3)	variable 4 (b4)
1	-1	-1	-1	-1
2	-1	-1	-1	+1
3	-1	-1	+1	-1
4	-1	-1	+1	+1
5	-1	+1	-1	-1
6	-1	+1	-1	+1
7	-1	+1	+1	-1
8	-1	+1	+1	+1
9	+1	-1	-1	-1
10	+1	-1	-1	+1
11	+1	-1	+1	-1
12	+1	-1	+1	+1
13	+1	+1	-1	-1
14	+1	+1	-1	+1
15	+1	+1	+1	-1
16	+1	+1	+1	+1

In a similar way, the matrix of experiments for 5, 6 or more factors can be built. It can be seen how in passing from one experiment to another, all – or at least most of the variables - are varied at the same time in a systematic way, contrarily to what happens in the one variable at a time approach. The levels can be represented in different ways but the one most widely used is to encode them to -1 (lowest level) and 1 (highest level), or simply as - and +. The same notation can be applied to qualitative factors, but this case -1 is not smaller than +1, it is only different.

A zero-level is also included, as a center, in which all variables are set at their intermediate value. Three or four experiments in the center should be included in factorial designs, for verifying whether any nonlinearity could be present and to estimate the experimental variance in order to assess the significance of the effects (parameters such as b1, b2, etc).

The sign for the interaction effect between variables is defined as the sign for the product of variables (table 2.4).

Table 2.4: 22 full factorial design with interactions

experiment number	variable 1 (b1)	variable 2 (b2)	interaction 1 and 2 (b12)
1	-1	-1	+1
2	-1	-1	+1
3	-1	+1	-1
4	-1	+1	-1

FRACTIONAL FACTORIAL DESIGN

When the number of factors increases, so does the number of experiments. In these cases, it is possible to perform only a part of the experiments (1/2, 1/4, 1/8) required by a full factorial design, and the design obtained is called fractional factorial design. As a consequence of performing only a part of the experiments, information on some or on all interactions may be lost. These experimental designs are also used to determine the collective effect of a combination of factors on the variance of a procedure, without analyzing in detail the individual contributions. Consistently to the notation already used for full factorials, these designs are often indicated as 2k-p, symbolizing that a only a fraction 1/2p of the experiments required for the complete design are to be performed. [53,54]. For example, a 2^{4-1} design is a design for 4 factors where only half of the experiments required by full factorial design (8 instead of 16) are performed. The following table shows the matrix of experiments for a fractional factorial design 2^{7-4}: in this case only 8 out of the 128 experiments needed according to a full factorial design with 7 factors have to be performed.

Table 2.5: fractional factorial design 2^{7-4}

experment number	variable 1 (b1)	variable 2 (b2)	variable 3 (b3)	variable 4 (b4=b12)	variable 5 (b5=b13)	variable 6 (b23)	variable 7 (b7=b123)
1	-1	-1	-1	+1	+1	+1	-1
2	-1	-1	+1	+1	-1	-1	+1
3	-1	+1	-1	-1	+1	-1	+1
4	-1	+1	+1	-1	-1	+1	-1
5	+1	-1	-1	-1	-1	+1	+1
6	+1	-1	+1	-1	+1	-1	-1
7	+1	+1	-1	+1	-1	-1	-1
8	+1	+1	+1	+1	+1	+1	+1

Of course reduction in the number of experiments comes with a cost: by using 2^{k-p} experiments to evaluate 2^k effects (model coefficients), then each terms is confused with other 2^{p-1}. For instance, considering the matrix of experiments in Table 2.5, it is possible to see that it was built from the matrix of experiments of a full factorial design of the same dimensions (2^3) by using the interaction terms to account for the sign combination of the other factors to be accommodated. Specifically, the signs for the variable 4 are the same as those of the interaction between variables 1 and 2, those for variable 5 as the ones of the interaction between factors 1 and 3, those for variable 6 as the interaction between factors 2 and 3 and the ones for variable 7 as the ternary interaction among variables 1, 2, and 3. Since only 1/16 of the original experiments are performed, each of these terms is confounded also with other 14 effects. When, as in the case reported in Table 2.5, the highest possible fraction of experiments is performed, the corresponding fractional factorial design is often used for screening and In model building assumption is made that only the terms corresponding to the main effect are significant, so that other confounded terms are neglected:

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + e \qquad (4)$$

In factorial or fractional factorial designs all variables are normalized between -1 and +1. For continuous variables, the scaling is made so that the original variables vary continuously within the interval from -1 to +1. Since all variables used in the model are normalized in this way, the relative change of a variable is directly related to the size of its regression coefficient. This means that if the model parameters have either a large positive or negative value the corresponding variable has a large influence on response.

IDENTIFY SIGNIFICANT EFFECTS

Once the design has been chosen and the experiments performed, to calculated the effect of the factors and their significance a simple procedure can be adopted, as far as full or fractional factorial designs are concerned. First of all, the offset b0 can be estimated as the average of the responses by summing the responses and dividing the sum obtained by the number of experiments carried out. On the other hand, calculation of all other coefficients is carried out multiplying point to point the column of the design matrix corresponding to the coefficient that has to be estimated by the column of the response and than taking the average of the results. Once the model coefficients are calculated, their statistical significance must be

assessed. To do so, at first the experimental variance s_y^2 must be estimated, e.g. by repeating the experimental design or by performing several measures (usually 3 or 4) in the central point of the design (at the test point). Then the standard deviation of the model coefficients (i.e. of the effects of the individual factors and interactions) can be calculated as:

$$s_{coeff} = \frac{s_y}{\sqrt{N}} \quad (5)$$

where N is the number of experiments in the design. Accordingly, the significance of the effect is estimated by means of a t test, and in particular, comparing the value of each coefficient (b1, b2, etc.) with the value *Scoeff***t*, where *t* is the critical value of Student's t at the opportune number of degrees of freedom and desired confidence level. A coefficient is significant and therefore the experimental variable or the interaction between the experimental variables is significant if the absolute value of the coefficient is greater than the value *Scoeff***t*. Depending on the sign of the coefficients, the most appropriate experimental conditions to minimize or maximize the response can be found in a relatively easy way.

Furthermore, when experiments are performed at the central point, it is also possible to validate the assumption of linearity of the model by comparing the predicted response at the center of the experimental domain, which is estimated by b0, with the actual measured values, again using a t test. In this case, b0 is compared to $t\frac{s_y}{\sqrt{C}}$, C being the number of replicate measurements at the center point.

RESPONSE SURFACE METHODOLOGY

The aim of these designs is to model the responses and to find the optimal combination of conditions. In these designs the factors are examined at more than two levels. The reason is that in the models, curvature of the response as a function of the factor levels is included, which requires testing of (at least) three levels. From a mathematical standpoint, this corresponds to a polynomial model which contains quadratic terms:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 + e \quad (6)$$

The differences with other multivariate optimization approaches such as the simplex one resides in the fact that models for the responses are built and that one assumes that the optimum of the method is situated in the experimental domain created by the selected extreme levels of the different factors.

It is a good way to graphically illustrate the relation between different experimental variables and the responses.

Box-Behnken designs (BBD) [55] are a class of second-order designs based on three-level incomplete factorial designs. For three factors, its graphical representation can be seen in two forms (A and B): A is a cube where there are a central point and the middle points of the edges (figure 2.1.a); B consists in a central point and three interlocking 2^2 factorial designs (figure 2.1.b).

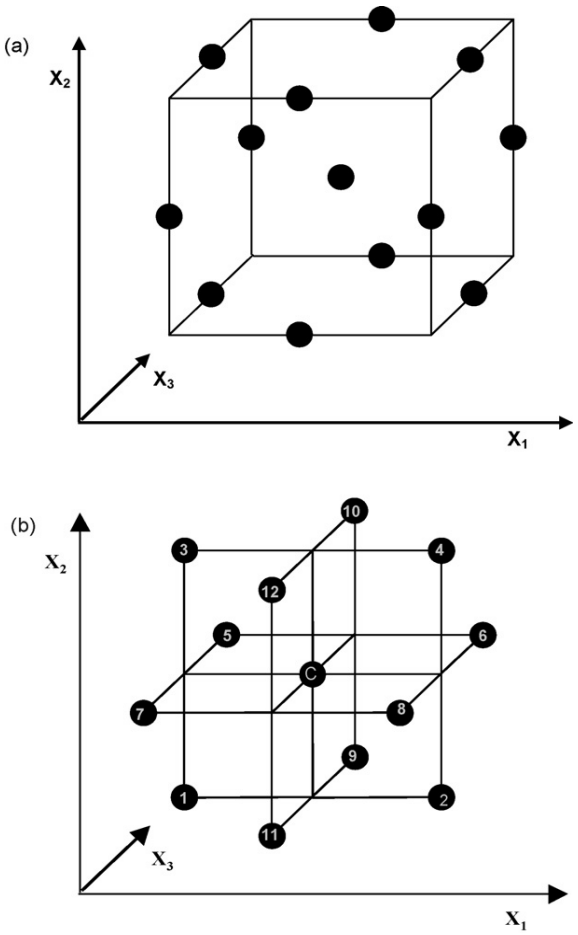

Figure 2.1: (a) the cube for BBD and three interlocking 2^2 factorial design (b) [56]

Table 2.1.6: Coded factor levels for a BBD of a three variable system

number of experiments	variable 1	variable 2	variable 3
1	-1	-1	0
2	+1	-1	0
3	-1	+1	0
4	+1	+1	0
5	-1	0	-1
6	+1	0	-1
7	-1	0	+1
8	+1	0	+1
9	0	-1	-1
10	0	+1	-1
11	0	-1	+1
12	0	+1	+1
Central	0	0	0
Central	0	0	0
Central	0	0	0

The number of experiments (N) required for the development of BBD is defined as $N=2*k*(k-1)+C0$, (where k is number of factors and C0 is the number of central points). The BBD is an efficient design, where the concept of efficiency is mathematically expressed as the ratio of the number of number of coefficients in the estimated model to the number of experiments. In fact, with a limited number of experiments it is possible to determine the linear terms and the quadratic terms. Another advantage of the BBD is that it does not contain combinations for which all factors are simultaneously at their highest or lowest levels. So these designs are useful in avoiding experiments performed under extreme conditions, for which unsatisfactory results might occur [56].

2.2 MULTIVARIATE CALIBRATION

Multivariate calibration techniques are widely used for the characterization of complex matrices, as, if experiments are carefully planned so that all the relevant sources of variability are spanned, they allow to reduce to a minimum or even completely bypass possibly

expensive chemical treatments and preventive separative operations. These operations are necessary when you use univariate methods of quantification, as complete selectivity of the measurement is assumed. In contrast to the univariate approach, which makes use, for the determination, of only one variable extrapolated from the entire set of those monitored (for example, an absorbance value at a wavelength corresponding to a maximum of a spectral profile), the multivariate approach allows to take advantage of the information obtained by the measurement operations [57].

The multivariate approach allows obtaining many benefits: for example, it is possible to build calibration models using techniques not perfectly selective, as the NIR spectroscopy, or build models for chromatographic and/or spectroscopic fingerprint.

Generally, a multivariate calibration involves the following steps:

1 defining the problem: selecting the property to determine;

2 selection of standards for the model construction: choose a sufficiently large number of samples that will guarantee a good statistical coverage of the calibration domain;

3 recording the signals (the variables): collect information about samples in a reproducible way;

4 building the regression model: finding the relation between response(s) and the variables measured on the samples (predictors);

5 validating the model: verifying the predictive ability of the model on "unknown" samples.

Concerning point 4), it may be opportune to define what regression is. In chemistry, regression is the search for a quantitative relation, which can be expressed by a particular mathematical equation, in order to be able to predict the value of one or more properties Y from the experimental measurements X; in other words, a function is sought that describes the relationship between two blocks of variables [58]. The regression is therefore, in general, the calculation of the unknown terms of an equation which, in the univariate case, takes the form:

$$y_i = b_0 + b_1 x_i + e_i \qquad (7)$$

where x is the independent variable (for example the concentration of a particular analyte), x_i is the value of this variable for the ith sample, y is the dependent variable (for example, the absorbance at a particular wavelength) and y_i is the value of this variable for the ith sample.

The terms b_0 and b_1 are the intercept (or offset term) and the regression coefficient, respectively, and represent the unknown terms that a regression problem aims to find. Finally, the term e_i is the residual for sample i, i.e. the error committed by the equation, which is defined as the difference between the predicted and the true values of y_i.
When the number of variables increases, equation 7 can be written in matrix form:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{e} \quad (8)$$

where X is the matrix that collects the measurements of each variable x for each sample (X collects a set of variables x characterizing objects, called predictors, for example the absorbances at different wavelengths), y is a vector which instead contains all the measured responses on the samples (for example, the concentrations), while b is the vector of regression coefficients, and e is a vector that contains the differences between the measured and predicted y [59].
In particular, Partial Least Squares Regression [60] is the most appropriate method to process data of spectral or chromatographic nature, in which a large number of variables are measured on a relatively small number of samples and in which the components of the multivariate signal (i.e., the X variables considered) are strongly correlated with one another.
The PLS algorithm compensates for the deep correlation commonly existing in the matrix of experimental data and that, generally, leads – in mathematical terms – to a so-called ill-conditioned problem, by operating a projections which results in a reduced number of orthogonal variables.
In detail, the PLS algorithm uses, for the calculation of the regression model, a new set of abstract variables (latent variables) computed as linear combinations of the original ones.
These variables are constructed to describe in the best way the correlation between the block of measured variables X and the block of the response variables Y (maximizing the correlation between X and Y), and at the same time to explain the structure and the variability of the original data (maximizing the variance of X and Y). In this way, the relevant information present in the matrix of the original data is compressed into a smaller number of latent variables, which also have the advantage of being orthogonal to each other, while the part of noise present in the data is collected in the last latent variables that will be discarded in the definition of the model. The regression is then made using this new set of variables, and the calibration of the model corresponds, in practice, to the search of the mathematical parameters

that define these latent variables. The reduction of the variables, from original variables to latent variables is sequential [61].

To describe the process by which the PLS components are calculated, in the following the single *y* case, i.e. the case where only one response is fitted, is considered: the predictors are then collected in a matrix *X* which is of size N (number of samples) x J (the number of original variables measured), while the response is represented by the vector *y* with dimensions N x 1. Assuming that both *X* and *y* are mean centered, the first PLS component t_1 is calculated as the one having the highest covariance with the y to be predicted:

$$\max_{\mathbf{w}_1}\left(\operatorname{cov}\left(\mathbf{t}_1,\mathbf{y}\right)\middle|\,\mathbf{t}_1=\mathbf{X}\mathbf{w}_1\ \&\ \left\|\mathbf{w}_1\right\|=1\right) \qquad (9)$$

where the normalized vector of weights w_1 defines the direction of the projection. The maximization of the covariance between *t* and *y*, dependents on the variance of *t* and depends on the correlation of *t* with *y*. This ensures that the noise present in the data may not be modeled (due to dependence on var(*t*)) and, at the same time, that the *t* chosen will be good to predict *y* (due to the dependence on corr(*t*, *y*)). Once t_1 is extracted, the procedure is repeated iteratively to calculate the subsequent components. In particular, at first the scores t_1 are regressed on *X* to obtain a vector of loadings p_1. The dyad t_1/p_1 is used to remove from the data matrix *X* the portion of variability which is accounted for by the first latent vector through a process which is called deflation:

$$\mathbf{E}_1=\mathbf{X}-\mathbf{t}_1\mathbf{p}_1^T \qquad (10)$$

Then the second component t_2 is calculated through the weight vector w_2, which is extracted according to a criterion analogous to equation 9, but involving E_1 instead of *X*.

All these operations are iterated until the desired number of latent variables is calculated; this number is usually estimated by cross validation (see section 2.5).

In the present thesis, the PLS algorithm was used for the quantitative determination of the parameters of quality of saffron (crocin, picrocrocin and safranal) and for the quantitative analysis of some indices required by law for the control of honey samples (reducing sugars, water and hydroxy methyl furfural).

2.3 MULTIVARIATE CLASSIFICATION: PARTIAL LEAST SQUARES DISCRIMINANT ANALYSIS (PLS-DA)

The qualitative methods of multivariate analysis are commonly known as pattern recognition. These techniques define the mathematical criteria that enable to quantify the similarity, usually in terms of distance, between two different samples or between a sample and a class.

A class can thus be defined as a collection of objects that meet certain specific criteria dependent on the problem at issue. In chemical terms, therefore, the recognition is made according to the results of the measurements made on different samples.

The fundamental problem of pattern recognition can also be represented geometrically, considering each pattern as a vector that describes a point in the hyperspace of variables, namely in the multidimensional space whose n axes correspond to the experimental variables monitored. In this way, each group of samples defines a set of points, a cluster. In this context, the purpose of pattern recognition, therefore, is to separate the hyperspace in a series of distinct and well-identified regions, in such a way that all the points that are located within them belong to the same class [62]. Depending on whether these classes are known a priori or not, it is possible to distinguish two different types of techniques: the first is called supervised learning or classification methods, while the other unsupervised learning or clustering methods. The term supervised highlights the use of a data set (training set), collected on samples the classes of which are known in advance, in order to extrapolate a classification rule. In many cases, to allow the evaluation of the real predictive capacity of the model developed, a second data set, also made of samples of known attribution (test set), can be used. Whenever a supervised learning strategy is adopted, it is possible to demonstrate that the decision rule minimizing the prediction error is called Bayes' rule, according to which a sample has to be assigned to the class it has the highest probability of belonging to.

From the applicative point of view, an important division which can be made is between discriminant (or pure) classification methods and class-modeling ones.

Discriminant techniques mainly focus on the differences between samples from different categories and divide the space of the variables in as many regions as there are classes: a sample is always assigned to one and only one of those [63]. The probability that a sample described by the vector x belongs to the class g - $p(g|x)$ - can be calculated, on the basis of Bayes' theorem, as the product of the probability of obtaining a vector of measurements x for

a sample extracted from the generic class g – the likelihood p(x|g) - and the probability, a priori, to observe a sample belonging to the same class g, $\pi(g)$, according to:

$$p(g|\mathbf{x}) = p(\mathbf{x}|g)p(g) \qquad (11)$$

Bayes' rule, therefore, involves two distinct phases: the calculation of the probability that a sample belongs to each class and the assignment of the sample to the class for which this probability of membership is higher. It is obvious that, depending on the method used, the results may be significantly different. Since it is not necessary to know the absolute value of this probability, but only for which class its value is maximum, sometimes it is preferable not to calculate directly the probability but a monotonic function of it, chosen in such a way as to simplify as much as possible the calculation. These functions are called classification functions:

$$f_g(\mathbf{x}) = f\left(p(g|\mathbf{x})\right) \qquad (12)$$

Once the parameters of these classification functions are calculated on the basis of training data, it is possible to define the boundaries which separate the regions of space corresponding to the different categories as:

$$f_{g1}(\mathbf{x}) = f_{g2}(\mathbf{x}) \qquad (13)$$

Modeling techniques instead are based on the recognition of the similarity between samples of the same category. Each class is modeled independently on the others and each sample can be assigned to one, more than one or none of them (asymmetric classification) [64].
In all cases described until now, the construction of the model consists of three basic steps:

1) Construction of hyperspace: projection of the set of samples in the multidimensional space defined by the original variables
2) Choice of the distinctive characteristics: reduction of dimensionality of the data matrix, targeted to the conservation of the maximum significant variance, and

graphical representation of the distribution of the samples within a low-dimensional space

3) Classification: formulation of a decision rule that allows the assignment of unknown samples to one of the distinct classes

Specifically, in the present thesis, classification studies of extra virgin olive oil, honey and saffron were completed using the discriminant approach by means of the algorithm PLS-DA (partial least square discriminant analysis).

As with the problems of multivariate calibration, to build a classification model means finding the best possible relationship between a multivariate independent matrix, whose ith row contains the values of the monitored variables on the i^{th} sample, and an array of qualitative responses, realized in such a manner as to include information relating to membership of the samples to one or any of the other classes considered. In this way, it is possible to use the traditional regression methods for the solution of non-quantitative problems [65]. The matrix of responses, the so-called dummy matrix, is consequently characterized by a number of rows equal to the number of analyzed samples and by a number of columns equal to the number of classes. Each row vector contains a value of 1 in correspondence to the column associated to the category of the relative sample while all the other values are instead equal to zero. The figure 2.2 shown the generic structure of a dummy matrix for n samples and g groups.

$$\mathbf{Y} = \begin{pmatrix} \mathbf{1}_{n_1} & \mathbf{0}_{n_1} & \cdots & \mathbf{0}_{n_1} \\ \mathbf{0}_{n_2} & \mathbf{1}_{n_2} & \cdots & \mathbf{0}_{n_2} \\ \vdots & \vdots & \ddots & \vdots \\ \mathbf{0}_{n_g} & \mathbf{0}_{n_g} & \cdots & \mathbf{1}_{n_g} \end{pmatrix}_{n \times g}$$

Figure 2.2: generic dummy matrix for n samples and g classes

Once the dummy Y matrix is built, its relation with the *X* block can be described using Partial Least Squares regression (see section 2.2). Accordingly, the use of PLS-DA allows building reliable classification models also for ill-conditioned problems [65].

2.4 DATA PRETREATMENT

Before applying chemometric methods, either for calibration or classification, it is often necessary to pre-treat the data. The term data pretreatment refers to a range of preliminary data characterization and processing steps. There are numerous methods for the pretreatment of chromatographic and spectroscopic data. The following are the main pre-treatments methods used for the pretreatment of chromatographic and spectroscopic data, with the aim of improving the performance of regression and classification models.

2.4.1 BASELINE CORRECTION: ASYMMETRIC LEAST SQUARE

The presence of a non-zero baseline in chromatographic signals can severely affect the bilinear modeling of the signals, by introducing additional components, and distort the similarity/dissimilarity relations among the samples. Therefore, baseline correction is an essential step when modeling such kind of signals. Baseline correction was carried out using the penalized asymmetric least squares algorithm proposed by Eilers [66]. The algorithm operates by estimating, for each chromatogram, the baseline *f* using a weighted least squares procedure in which the value of the weights is iteratively changed until convergence. In particular, for each chromatogram, the solution is sought as the baseline *f* which minimizes the following cost function:

$$L = \sum_{i=1}^{NP} w_i \left(y_i - f_i\right)^2 + l \sum_{i=1}^{NP} \left(\mathrm{D}^2 f_i\right)^2 \qquad (1)$$

where y_i and f_i are the value of the i^{th} point of the experimental signal and of the estimated baseline, respectively, w_i is the weight associated to that point and *NP* is the total number of

points. The second term in equation 1 is introduce to govern the smoothness of the solution, through the regularization parameter λ: $\Delta^2 f$ indicates that second-order differences are used to evaluate the roughness of the solution. Baseline estimation is an iterative step: initially, all points are given unitary weight and a first approximation of f is calculated. Then, weights are asymmetrically updated so that only those points which are below the approximated solution will contribute relevantly to the definition of the baseline at the successive iteration:

$$\begin{cases} w_i = p & \text{if } y_i > f_i \\ w_i = 1 - p & \text{if } y_i \leq f_i \end{cases} \qquad 0 < p < 1 \tag{2}$$

To impose that all data points with positive deviation from the approximation *f* exert a very small influence on the baseline approximation, usually *p* is given the value 0.01 or less). Once the baseline is approximated in a satisfactory manner, it can be subtracted from the studied signal.

2.4.2 ALIGNMENT OF CHROMATOGRAPHIC PEAKS: INTERVAL CORRELATED SHIFTING (ICOSHIFT)

After correcting the baseline, it was necessary to pretreat further chromatographic signals to ensure that the peaks of the analytes were aligned. Retention time alignment is useful for peak identification and quantitation, but is especially important as a pre-treatment step before the application of classification models like PLS-DA. Indeed, in order for PLS-DA to work well, the same underlying process must be associated to the same variables in all the samples. In the case of chromatographic data, this implies that retention time, for the same compound, must be invariant across all samples. If this condition is not met, as it is often the case in real world experiments, the predictive ability of classification model and the chemical interpretation of the results can be compromised [67]. Misalignment problem can be overcome by using alignment algorithm; in particular, in the present study, *Interval Correlation Optimized Shifting* (*i*coshift) algorithm was used for aligning HPLC-DAD data [68]. *i*coshift divides spectra into segments, and aligns these to the corresponding segments of a reference spectrum. The alignment is performed by shifting the segments sideways to maximize their

correlation. In practice, this involves calculating the crosscorrelation between the segments by a fast Fourier transform (FFT) engine that aligns all signals of a data set simultaneously.

2.4.3 VARIABLES SELECTION: BACKWARD INTERVAL PARTIAL LEAST SQUARE (BiPLS)

PLS and PLS-DA algorithms were used for the construction of quantification and classification models. Before constructing a model, in addition to the correction of the base line and the alignment of the chromatographic peaks, it can be useful to reduce the number of variables.

The predictive capability of a multivariate classification or regression model can be affected by the presence of a large number of variables: not all the points that constitute the instrumental profile carry relevant information, so that a selection of portions of the signal can significantly improve the results. For this purpose, in the present thesis the technique Backwards Interval PLS (Bi-PLS) coupled to Genetic Algorithms (GA) was used [69].

The BiPLS algorithm allows to calculate local PLS models using as predictors only variables contained in equidistant intervals in the matrix *X*. In particular, in its backward implementation, at every iteration, the calibration is carried out using a data matrix built by eliminating from the entire instrumental profile the selected intervals. For each model, a cross validation step, with a certain number of cancellation groups, is executed: the interval whose elimination results in the minimum value of the RMSECV (see section 2.5) error, is the one which is finally excluded before the algorithm proceeds with the next iteration. The routine is thus interrupted once a minimum value of selected variables is reached. However, in many practical cases, to avoid problems related to the possibility that a peak is divided between two intervals, the entire procedure described is repeated by gradually increasing the number of intervals up to a fixed maximum value. At the end of these repetitions the frequency of selection of the variables is evaluated, on the basis of which the final set of variables to store is defined [69].

2.4.4 VARIABLES SELECTION: GENETIC ALGORITHMS

To finalize the selection of variables, it is essential to apply to the data matrix, previously reduced by biPLS, characteristic genetic algorithms (GA). The coupling between the two procedures is necessary since, due to the enormous amount of monitored variables, it is not possible to use only GA [70]. In particular, genetic algorithms constitute an optimization method which is based on biological evolution. By analogy, any possible solution provided for a specific problem is called chromosome, while each iteration of the computational procedure is defined generation. More than one solution at a time is tested, so that in general one speaks of a population of chromosomes. The aim of the application of genetic algorithms is to evolve the population along several generations to find the best solution to the problem.

To determine the quality of these solutions, the concept of fitness, i.e. any mathematical criterion that determines the goodness, is introduced. Usually, the fitness is defined as the minimum error committed by the model during cross-validation. As this problem concerns the selection of the variables, each chromosome will correspond to a binary vector of length equal to the number of points that constitute the whole signal and will contain zeros in the positions of the variables to not be selected and ones elsewhere. The algorithm is initialized by generating at random a number of possible solutions, or of groups of variables: these variables are then used to build the corresponding regression or classification models, and the relative error in cross validation is calculated. Once the chromosomes are sorted in descending order of fitness, the algorithm proceeds by using two different operators, crossover and mutation, which allow at the same time the exploitation of the "genetic" material in the actual population and the exploration of new possible solutions.

In particular, the crossover modifies the structure of two chromosomes selected operating an exchange in blocks between their internal values. The mutation, instead, operates by inverting the values of each position of the chromosome with a probability equal to 0.1%. Consequently, for each iteration two new chromosomes will be built, the quality of which should then be evaluated. The solutions that are characterized by a better fitness compared to those present in population at that particular generation will replace the worst ones [71].

The entire procedure is then repeated for a fixed number of generations, the end of which, the chromosome, or the group of variables, corresponding to the higher fitness is selected as the best. To ensure high consistency of results, generally, a genetic algorithm is not applied only once to a matrix of data, but for a sufficiently large number of times, equal to 100 in our case,

so that, in the end, one will include in the final model only the variables most frequently selected.

2.5 VALIDATION OF CHEMOMETRIC METHODS

The validation of the developed models, for the study of multivariate classification or quantification, is of uttermost importance for the evaluation of the reliability of their performances. To this purpose, there are different techniques useful for the control and the determination of the quality of the results obtained by chemometric analysis, which differ according to the type of problem in exam and samples available. More generically, the validation procedures are to be able to provide information of two kinds: in fact they allow to obtain both indications on how to maximize the sensitivity of the model and its effective capacity to characterize unknown samples, confirming the reliability and ensuring that their solutions are generalizable [72].

The increase in the quality of a chemometric model depends on the possibility to minimize the error in the prediction of a certain type of response, which for the i^{th} sample can be determined according to the following equation:

$$e_i = y_i - \hat{y}_i \qquad (14)$$

where $\hat{y}_i$ and y_i represent the value of the response predicted by the built model and its reference value, respectively. However, in sets of samples very different from one another, a common situation in the case of multivariate calibration and classification, it is not much important reducing the prediction error on the individual object, but rather the average error made on the totality of the validation group (test set). This error is defined RMSEP, Root Mean Square Error of Prediction; if n_{new} samples, different than those used for model construction, are used to validate the model, RMSEP is calculated as:

$$RMSEP = \sqrt{\frac{\mathbf{e}_p^T \mathbf{e}_p}{n_{new}}} \qquad (15)$$

where e_p is a vector containing the differences between the reference and the predicted values for all the test set samples.

Similarly, it is possible to determine the error of the model in the calibration phase, the RMSEC, Root Mean Square Error of Calibration, according to the following equation:

$$RMSEC = \sqrt{\frac{\mathbf{e}_c^T \mathbf{e}_c}{n - df}} \quad (16)$$

In this case, e_c is formed by the residuals associated with the n samples on the basis of which the model has been realized, while df is equal to the number of degrees of freedom lost in the estimation of its parameters. In addition to the classical procedure of external validation, it is possible perform a kind of systematic resampling of all available data for subsequent evaluation of different sets, this operation is called cross-validation. It acts by splitting the set of samples available in a number k of so-called cancellation groups. Iteratively, each of these sets is not included in the calibration phase and it is used as a validation set. In total, therefore, k different models will be constructed, so that eventually the RMSECV, Root Mean Square Error of Cross Validation, is evaluated as:

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{n} \left(y_i - \hat{y}_{i,cv}\right)^2}{n - df}} \quad (17)$$

where y_i and $\hat{y}_{i,cv}$ are the reference value of the response for the ith sample and its predicted value from the model built when the sample is left out in one of the k cancellation groups, respectively, while the other terms have the same meaning as in equation 17.

Generally, while external (test set) validation is used to evaluate the predictive ability of the models, cross-validation is employed to estimate the optimal model complexity, i.e. the number of components needed for the construction of the best model [73].

CHAPTER 3

EXTRA VIRGIN OLIVE OIL: GEOGRAPHICAL TRACEABILITY OF SABINA PDO

3.1 INTRODUCTION

In recent years, the issues of food quality and safety have received a special attention both from the producers and the consumers [74-77]. In this context, a key role is played by the identification, authentication and traceability of foodstuff [78-80]. In particular, while the former terms indicate, in general, to what extent the products comply to what declared on the label and to the law, the latter concentrates on the ability to track any food through all stages of production, processing and distribution. Based on this definition, it is evident how the possibility of well-enacted food traceability protocols could on one hand represent an effective way of managing the risks connected to foods and feeds, for instance by allowing to quickly isolate contaminated products and prevent them from reaching consumers, or guiding targeted withdrawals when needed. On the other hand, since the largest share of traceability issues is related to the verification of the geographic, species or production origin of the goods, it can also represent a solid ground to protect local and/or regional foods, to help producers obtain a proper price for their authentic products, and to avoid the unfair market

competition by products, which may be of inferior quality. In the framework of the European Union, this necessity of protecting and promoting the names of traditional, regional and quality foodstuff has been enforced through the introduction of the geographical indication marks: protected designation of origin (PDO), protected geographical indication (PGI), and traditional specialties guaranteed (STG) [81,82]. In particular, the attribution of a geographical indication mark certifies that the quality of the foodstuff is significantly or exclusively determined by its geographical environment, including natural and human factors, and that its production, processing and preparation took place within the determined geographical area [83].

Extra virgin olive oil (EVOO), due to its chemical composition and characteristics, in particular its nutritional and biological properties [84,85], represents a high quality and value-added product, whose price can be even 6-7 times higher than that of other edible vegetable oils. Given the specificities of its production, EVOO is one of the foods whose quality is most closely related to the terroir, i.e. "the set of special characteristics that the geography, geology and climate of a certain place, interacting with the plant's genetics, express in agricultural products" [86], and consequently already many oils from different European countries (mainly Italy, Spain, Greece, France and Portugal) are protected by the PDO mark. Among these, Sabina EVOO is the first Italian oil to have gained the PDO status, in 1996 [87]. It is produced in the homonymous territory in the Lazio region, which is part of the provinces of Rome and Rieti and owes its name to the fact that this area was originally occupied by the ancient Sabines tribe; indeed, Sabina has been an oil producing region since 7^{th}-6^{th} century B.C. Nowadays, the production disciplinary for the PDO [88] provides that Sabina oil should be made from the following olive varieties, either combined or singularly: "Raja", "Leccino", "Frantoio", "Carboncella", "Pendolino", "Olivastrone", "Salviana", "Rosciola", "Olivago" and "Moraiolo". Its sensory characteristics are defined as: golden yellow color, with greenish tinges when very fresh, fruity aroma and smooth, even, fruity, fragrant, sweet, flavors, bitter when very fresh. As a consequence of their provenance and of the reported organoleptic characteristics, Sabina PDO oils are considered high quality products and due to their higher market price can be subjected to frauds, such as the addition of cheap oils and/or the marketing of oils from other regions under the same name. However, from the quality control standpoint, the traceability system enacted so far by the European authorities operates mainly a priori, defining in detail the percentages of specified cultivar olives, cultural practices, circumscribed geographical production areas, chemical and sensorial properties needed to obtain the PDO label in the production disciplinary, and relying only on the inspection of the

production registries for the verification of the authenticity of the products. On the other hand, at present no analytical indices exist which could be measured on the final product, allowing, a posteriori, to distinguish it from other oils of lesser value. Accordingly, the possibility of building traceability models by coupling chemometric classification methods to the analytical characterization of the products (often by fingerprinting techniques) is becoming more and more fundamental for the verification of the authenticity of foods with geographical indication marks [89-94]. In this framework, variations in the phenolic composition of virgin olive oils related to cultivars, ripening and production techniques were already reported in the literature [95-97], suggesting that the chromatographic fingerprinting of this fraction could be a valuable tool for tracing the origin of the product. On the other hand, considering the increasing attention to the potential health benefits associated to different classes of biophenols (for instance, prevention against cardiovascular diseases, immunoregulation diseases, or asthma [98-100]), the possibility of relating the higher quality of the product to the phenolic composition would be of utmost importance for its characterization. Therefore, aim of the present study was to verify the potential of using the chromatographic fingerprinting of the phenolic fraction coupled to chemometric data processing for the authentication of the PDO Sabina oils. To this purpose, at first Partial Least Square-Discriminant Analysis (PLS-DA) was used to process the chromatographic profiles recorded at three selected wavelengths (254, 280 and 340nm), while in a second stage, mid-level data fusion was used to integrate portions of the signals at the different wavelengths, selected by a successive application of backwards interval Partial Least Squares (biPLS) and genetic algorithms (GA), in order to achieve better classification performances and easier interpretability.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLES

Sabina samples (20 oils) were all taken from different oil mills in various place of the region, covering as representatively as possible the whole production area in terms of geographical

position, maturation olives and climate conditions and also of manufacturing techniques employed: traditional (press) or modern (two, two and half, and three phases decanters). Samples coming from other origins (here, for the sake of simplicity, labeled "not Sabina", 57 oils) were provided by the chemical laboratories of the Customs Agency (Rome, Italy). These samples were collected from the producers within 10 days of production, and immediately frozen and stored at -20° C in their laboratories, and carried in our laboratory by mean of an ice bag.

3.2.2 OPTIMIZATION OF THE EXTRACTION OF THE PHENOLIC FRACTION FROM OLIVE OIL

To isolate the phenolic fraction from the olive oil matrix, a rapid liquid-liquid extraction (LLE) procedure was used: 1 g of oil was diluted with hexane and extracted with water/methanol solution. The optimal extraction conditions were optimized using an experimental design. In particular, considering the experimental domain and the number of controlled factors, a two level full factorial design was chosen. Indeed, after some preliminary screening experiments, it was observed that four factors only might relevantly affect the recovery: the volume of hexane used to dilute the sample, the composition and volume of the water/methanol solution, and the extraction time. Therefore, a 2^4 factorial design was used, controlling each factor at the levels reported in Table 3.1. The sum of the areas of all peaks within the phenolic fraction was used as the response to be optimized.

Table 3.1: Experimental domain for the optimization of LLE

Level	**Hexane vol.**	**MeOH:H_2O Ratio**	**MeOH:H_2O vol.**	**Extraction time**
+1	1 mL	80:20	4 ml	1 min
-1	2 mL	60:40	6 ml	3 min

When analyzing the results, none of the interaction terms and only two of the main effects resulted to be significant: the composition of the water/methanol solution and the extraction time, their optimal values being MeOH/H_2O 80:20 v/v and 3 min, respectively. Since the effect of the other two factors was shown to be not statistically relevant, their values were fixed at the level which resulted more advantageous in terms of rapidity and costs (1 mL hexane and 4 mL hydroalcoholic solution, respectively). Accordingly, the final extraction procedure resulted to be the following: 1 g of olive oil was diluted with 1mL of hexane and then extracted with 4mL MeOH:H_2O 80:20 v/v for 3 minutes. Then, the hydroalcoholic extract containing the analytes was filtered and evaporated to dryness in rotavapor at 30 °C; the residue was dissolved in 200 µL of MeOH:H_2O (50:50, v/v) and 20µL of the solution were injected into the HPLC. This extraction showed a good reproducibility (RSD < 5%), and it was used for the analysis of all EVOO samples.

3.2.3 HPLC-DAD ANALYSIS OF THE PHENOLIC FRACTION

For the chromatographic analysis of olive oil samples, biochanin A was used as internal standard (10 µg/g olive oil). The extracts prepared according to what described in section 3.2.2 were analyzed by HPLC-DAD with a Thermo Quest Spectrasistem LC (Thermo Fisher Scientific, Waltham, MA) equipped with a P4000 pump, a UV6000 UV-Vis Diode Array Detector, and a SN4000 interface to be operated via a personal computer. Extracted compounds were separated using an Eclipse XDB-C18 analytical column (4.6x250mm, 5µm particle size; Agilent Technologies, Santa Clara, CA) protected by a guard cartridge of the same packing, operating at 25° C. The mobile phase consisted of a binary solvent system using water (solvent A) and methanol (solvent B) both acidified with 0,1% formic acid kept at a flow rate of 0.8 mL/min. The gradient program started with 90% eluent A and 10% eluent B. This percentage was maintained for 5 minutes and eluent B was ramped linearly to 20% in 5 min and kept constant for 10 min; eluent B was ramped again linearly to 30% at 30 min and to 70% at 45 min. The chromatographic profiles were collected at 254, 280 and 340 nm.

3.2.4 IDENTIFICATION OF POTENTIAL PDO MARKERS BY HPLC/ESI-MS

For the qualitative analysis and, in particular, to identify which compound could be most significant for characterizing the PDO Sabina, HPLC/ESI-MS analysis was carried out. In particular, selected portions of the eluate from the HPLC-DAD system were sampled at the detector, evaporated to dryness under N_2, dissolved in 50µL of MeOH and injected into the HPLC/ESI-MS system. In particular, a Perkin-Elmer series 200 (Norwalk, CT, USA) liquid chromatograph, equipped with a binary pump and a vacuum degasser was used. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was conducted using a QTRAP™ quadrupole linear ion trap (QLIT) instrument equipped with a TurboIon-Spray (TISP) interface (Applied Biosystems/MDS Sciex, Concord, ON, Canada) operated in positive and negative ion modes. The whole apparatus was controlled by Analyst software (Applied Biosystems/MDS Sciex). The chromatographic column was an Alltima-C18 (2.1x250mm, 5µm particle size; Alltech, Deerfield, IL, USA). The mobile phase and gradient program were the same described for HPLC-DAD analysis (section 3.2.3) while flow rate was 200µL/min. Each sample was analyzed twice, once in positive ionization mode and once in negative ionization mode in the ranges 100-850 m/z (MS) and 90-850 m/z (MS/MS) using an information-dependent data acquisition (IDA) protocol.

3.2.5 SIGNAL PRE-PROCESSING

Since chromatographic signals could be affected by unwanted sources of variability, which could result in baseline contributions and shifts in the position of peak maxima, prior to the classification analysis a data preprocessing step was carried out. Baseline correction and peak alignment are essential steps, and in this study they were carried out using the *penalized asymmetric least squares* algorithm and *interval correlation optimized shifting* (*i*coshift) algorithm (described in the previous chapter, Sections 2.4.1 and 2.4.2)

3.2.6 CLASSIFICATION

Classification models were built using Partial Least Squares-Discriminant Analysis (PLS-DA) [65]. PLS-DA is a discriminant classification technique based on Partial Least Squares regression (PLS), where the dependent matrix **Y** is a binary-coded dummy matrix encoding information about class belonging. In particular, the number of columns of **Y** is the same as the number of categories in the classification problem and each row of the matrix is built as a vector of all zeros except for the component corresponding to the class of the samples, which has a value of 1. Since PLS predictions are real valued, for classification the sample is assigned to the category corresponding to the highest value of the predicted Y component.

3.3 RESULTS AND DISCUSSION

The 77 oil samples considered in this study where characterized by recording the chromatograms of the phenolic fraction, after liquid-liquid extraction, at 254, 280 and 340nm, as described in sections 3.2.2 and 3.2.3. The chromatographic profiles of each sample were considered as a fingerprint of the olive oils to be used, together with classification techniques, in particular PLS-DA, for building a traceability model able to discriminate Sabina samples from other oils. To this purpose, at first classification models were built considering each of profiles recorded at the different wavelengths separately, while in a further stage mid-level data fusion was used to integrate the information coming from the various chromatograms. However prior to model building, it was necessary to preprocess the data, as described in Section 3.2.5. In particular, baseline correction was performed using the penalized asymmetric least squares algorithm, testing different combinations of p and λ, and selecting the optimal value of the parameters by visual inspection of the resulting pretreated; the best combination resulted to be p=0.001 and $\lambda=10^5$. Successively, icoshift was used to align the signals. For each wavelength, a preliminary warping by coshift on the whole signal was performed using the median chromatogram as the target. Then, for the successive icoshift run, 14 intervals unevenly spaced were manually selected and a maximum shift of 100 data points was allowed, using as the target a signal built by taking, for each retention time, the maximum

intensity over all samples. The results of these two pretreatment can be visualized in Figure 3.1 on a time window selected as example.

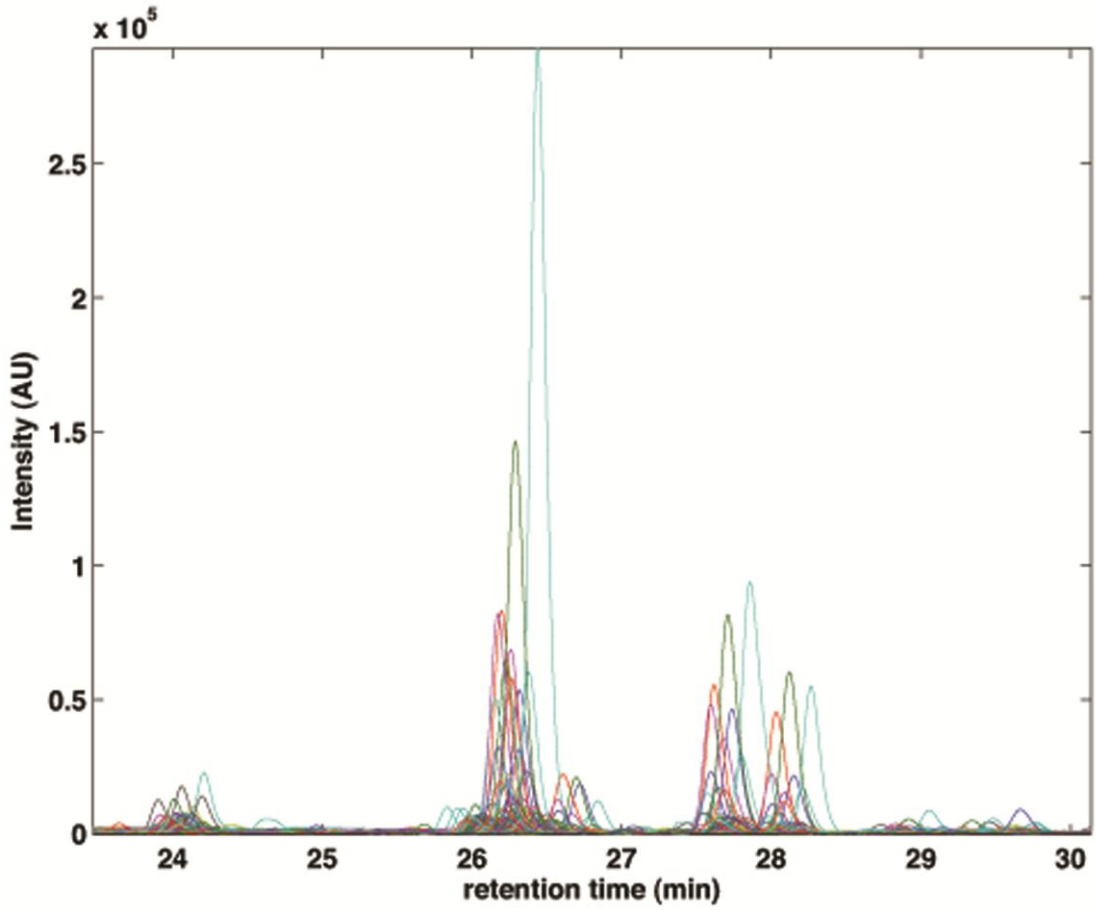

Figure 3.1A: chromatograms recorded at 340nm, without pretreatments

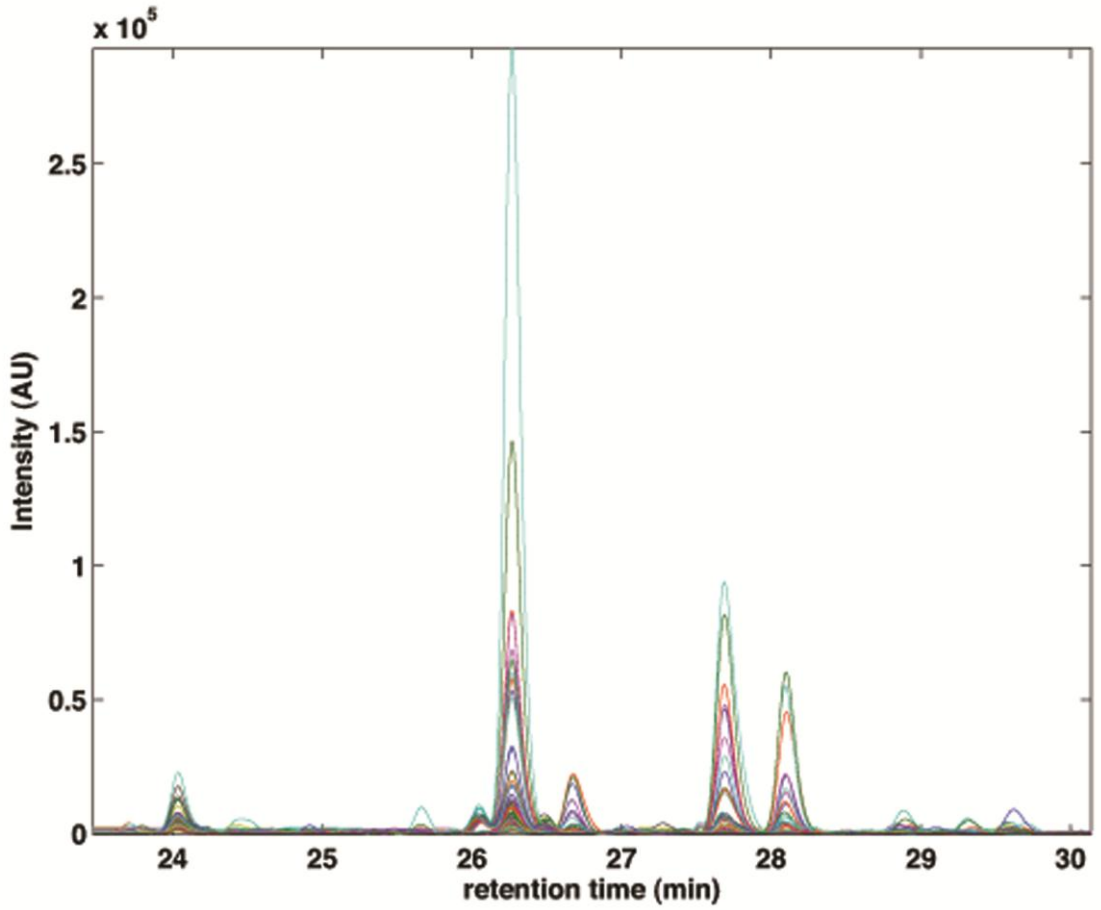

Figure 3.1B: chromatograms recorded at 340nm, after pretreatments

After baseline correction and alignment, the fingerprints were normalized by dividing the intensity at each data point by the area of the internal standard.

In order to have a set of independent samples to validate the predictions of the developed classification models, data were then split into training and test sets by means of the duplex algorithm [73]. Duplex algorithm was chosen as it keeps the same diversity within two sets: it operates by first finding the two pairs of farthest samples to constitute the basis of the training and test set and then by adding, alternatively, the sample which is most different to those already present in the set. In this context, difference is defined by the so-called *maximin* criterion: the most different sample is the one having the maximum value of the minimum distance to all the individuals in the set. In the present study, to guarantee that both classes were properly represented, duplex algorithm was separately applied to each category with a splitting ratio of 2:1. Moreover, in order to use the same training/test division throughout the

study, the selection algorithm was operated on a matrix obtained by concatenating the scores along the significant principal components extracted from the three data set corresponding to the signals at 254, 280 and 340 nm. Accordingly, 50 samples (13 from Sabina and 37 from other origins were selected as training set and the remaining 27 (7 from Sabina and 20 from other origins) left out as external validation set.

3.3.1 PLS-DA ANALYSIS ON INDIVIDUAL DATA MATRICES

At first separate PLS-DA models were built on the individual data matrices containing the chromatographic profiles recorded at 254, 280 and 340 nm, after mean centering. Selection of the optimal complexity of the latent variable models was made on the basis of the minimum classification error in 5-fold cross-validation. The results obtained are reported in Table 3.2.

Table 3.2: Results of PLS-DA modeling on individual data sets without variable selection

Wavelength	**LVs**	**%Correct Class. Calibration**		**%Correct Class. CV**		**%Correct Class. Prediction**	
		Sabina	Not Sabina	Sabina	Not Sabina	Sabina	Not Sabina
254nm	4	92,3%	89,2%	84,6%	75,7%	71,4%	75,0%
280nm	3	92,3%	81,1%	84,6%	78,4%	57,1%	75,0%
340nm	3	84,6%	83,8%	80,4%	83,1%	57,1%	85,0%

It can be observed from the Table that in all cases rather parsimonious models are obtained, and that in general a rather good classification ability is observed both in calibration and cross-validation. However, when the trained model are applied on the external test set, the performances are significantly worse, especially for the class Sabina. Based on these results, in order to improve the classification ability of the models, by including only those parts of the signal carrying the discriminant information, variable selection was carried out by means

of a sequential application of backward interval-PLS and genetic algorithms (biPLS-GA) [69]. The biPLS step, indeed, allows to operate a preliminary variable reduction, so that the genetic algorithm can operate of a number of predictors which is low enough (usually <250) to minimize the risk of overfitting. In detail, biPLS was algorithm was run 21 times, changing the interval size at each iteration (each of the data matrices, after autoscaling was divided in a number of intervals ranging from 25 to 45) and the predictors most frequently selected over the runs, were pooled to constitute the data set on which GA variable reduction was operated. Leardi's hybrid GA [101] was used with a population of 30 chromosomes and probabilities of cross-over and mutation of 0.5 and 0.01 respectively, on autoscaled data. Classification error in 5-fold cross-validation was used as the fitness function and the optimal number of evaluations was chosen on the basis of randomization tests, as the one minimizing the risk of selecting irrelevant variables and overfitting [101]. The results of PLS-DA analysis after variable selection are reported in Table 3.3.

Table 3.3: Results of PLS-DA modeling on individual data sets after variable selection by biPLS-GA

Wavelength	**LVs**	**%Correct Class. Calibration**		**%Correct Class. CV**		**%Correct Class. Prediction**	
		Sabina	Not Sabina	Sabina	Other origin	Sabina DOP	Sabina
254nm	4	92,3%	91,1%	84,9%	75,7%	85,7%	77,0%
280nm	3	92,1%	86,5%	88,5%	85,0%	85,7%	85,0%
340nm	3	92,3%	91,9%	83,5%	85,0%	85,7%	85,0%

It can be seen from the Table that variable selection significantly improved the classification ability of the models, especially for the class Sabina, which is the one of interest. Moreover, better results are obtained on the data sets at 280 and 340 nm with respect to the one at 254 nm, as it could be expected, since the signals at higher wavelengths are more selective and

less affected by interferents. The chromatographic regions which were chosen by the biPLS-GA algorithm are shown in Figure 3.2.

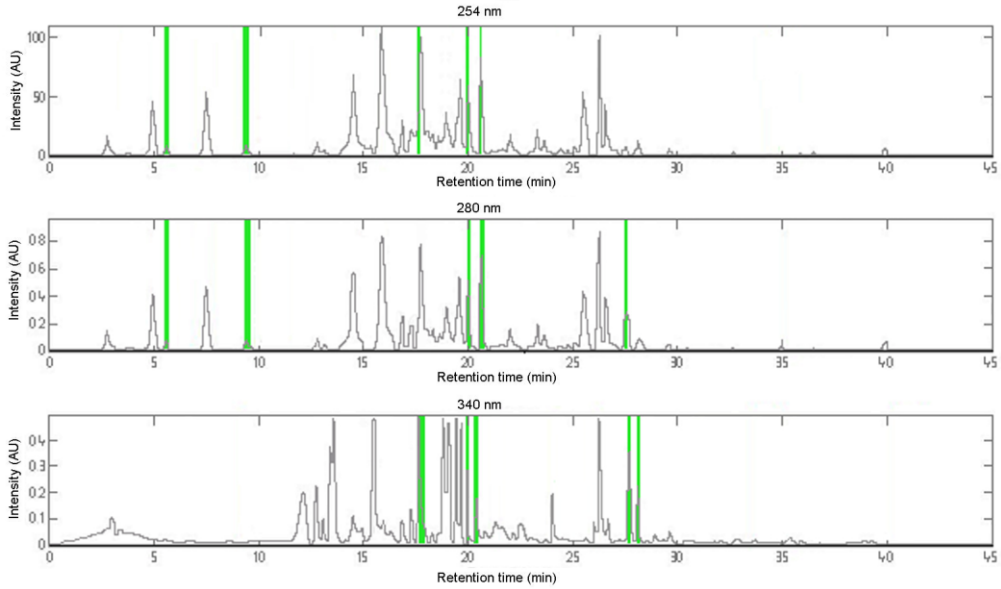

Figure 3.2: chromatographic regions (highlighted in green) which were chosen by the biPLS-GA algorithm

It can be observed from the Figure that biPLS-GA selected in all cases meaningful regions of the signals, corresponding to specific chromatographic peaks. Moreover, selection of the peaks was in most cases consistent at the different wavelengths: this is a further indication that the variables were not selected on the basis of chance correlations with the desired response.

3.3.2 DATA FUSION

To verify whether the classification ability of the PLS-DA models could be further improved by integrating the information from the signals at the different wavelength, a data fusion strategy was considered. In particular, to take advantage of the better results obtained after variable selection on the individual matrices, the so-called mid-level fusion strategy was adopted. In mid-level data fusion, the concatenation occurs at the level of the features extracted from the individual matrices. In particular, in the present study, for each data set, the variables selected after biPLS-GA were concatenated to form the final fused matrix: since three individual data set were available, all the possible binary combinations, plus the one involving all the three wavelengths together were tested and the results are reported in Table 3.4.

Table 4: Results of PLS-DA modeling on individual data sets after variable selection by biPLS-GA and mid-level data fusion

Wavelength	**LVs**	**%Correct Class. Calibration**		**%Correct Class. CV**		**%Correct Class. Prediction**	
		Sabina	Not Sabina	Sabina	Other origin	Sabina DOP	Sabina
254nm+280nm	4	92,3%	91,9%	91,2%	88,0%	85,7%	80,0%
254nm+340nm	1	92,3%	86,5%	88,8%	85,4%	85,7%	85,0%
280nm+340nm	3	100%	91,9%	91,2%	91,4%	85,7%	90,0%
254nm+280nm+340nm	2	100%	97,3%	87,7%	85,0%	85,7%	85,0%

When looking at the Table, it is possible to observe that in almost all the cases, the classification results after data fusion are the same as the best one obtained on individual matrices after variable selection. This outcome is not completely unexpected as it was already shown how the peaks selected by biPLS-GA at the different wavelengths were in most cases

the same, and so the selected features which were fused carry almost identical information. The only exception was the data set resulting from the fusion of the variables selected at 280 nm and 340 nm, which allow to improve the sensitivity of the models, as the correct classification rate for the not Sabina oils increased to 90%.

3.3.3 IDENTIFICATION OF POTENTIAL TRACEABILITY MARKERS FOR THE PDO SABINA

To interpret the results obtained after variable selection in terms of chemical species which could carry a discriminant information and, hence, which could be used as traceability markers for the PDO Sabina, HPLC/ESI-MS analysis was carried out, as described in Section 3.2.4. The analytes corresponding to the peaks selected by biPLS-GA were identified by tandem MS either in positive or negative mode and they are listed in Table 3.5.

Table 3.5: Compounds identified by HPLC/ESI-MS

Retention time	**Compound**	**Ion mode**[a]	**m/z**	**Fragments**[b]	**Identification**[c]
5.4	vanillic acid	negative	167.1	108.0(100);151.8(10)	A
9.1	p-coumaric acid	negative	163.1	119.1(100);167.1(27); 91.1(13)	A
18.2	luteolin	negative	285.2	133.2(100);107.2(20); 151.2(17)	A
19.9	pinoresinol	positive	359.1	359.1(100);327.1(10)	A
21.0	acetoxypinoresinol	positive	417.4	417.4(100);358.4(10)	B
26.8	apigenin	negative	269.0	117.0(100);107.0(17); 151.0(12)	A
27.9	methoxyluteolin	negative	299.4	299.4(100);199.4(25); 191.4(20)	B

[a] collision energy was 25eV for negative ion mode and 30eV for positive ion mode;

[b] the relative abundances are shown in brackets;

[c] identification was based on: (A) retention time and MS data consistent with those of authentic standard; (B) MS data consistent with literature.

3.4. CONCLUSIONS

The results obtained in the present study suggest that the phenolic composition of olive oils can constitute a reliable basis to discriminate PDO oils, when coupled to chemometric classification techniques. In particular, it was shown that by selecting specific portions of the chromatograms recorded at 280 nm or 340 nm, it was possible to correctly classify about 85% of samples in external validation. Moreover, when the information from these two wavelengths was combined through a mid-level data fusion strategy, the specificity of Sabina with respect to the oil of the other origin raised to 90%. The use of variable selection strategies, together with improving the correct classification rate of the models, allowed also to identify a reduced set of predictors carrying the greatest part of the discriminant

information, which could then be thought of as a potential traceability markers for the PDO Sabina. Identification of these analytes by HPLS-MS analysis showed that the substances which contributed the most to the discrimination of Sabina PDO from other oils are all phenols of high nutritional and biological value: vanillic acid, *p*-coumaric acid, luteolin, pinoresinol, acetoxypinoresinol, apigenin, methoxyluteolin.

CHAPTER 4

HONEY: GEOGRAPHICAL AND BOTANICAL TRACEABILITY

4.1 INTRODUCTION

Honey is a food consumed all over the world and appreciated as a quick source of energy, but also for its antibacterial and antioxidant activity. Commercially there are both mono-floral honeys (mainly made from a single botanical species) and polyfloral honeys (obtained from different plant species). Each one has its own composition and organoleptic characteristics derived from the type of flora worked by bees that make the honey undoubtedly a product linked to the area of production.

Honey is a food often adulterated; moreover, differences in price and quality are present between honeys of European countries, China or South America, but there are also differences between honeys of the various European countries or even between regions within the same country. The European Union Commission is encouraging the development of new analytical methods for monitoring and verifying the quality of the different honeys and for identifying their geographical origin. The product label must show the country or countries of origin where the honey has been produced. When the honey originates in more Member States or third country the indication may be replaced by one of the following sentences: "blend of CE honeys", "blend of non-CE honeys 'or' blend of honeys not originating in the CE". In addition,

the botanical origin of honey must also be indicated on the label, so it must be declared whether a honey is polyfloral or it is obtained from a specific species of plant.

The method that is currently used in the determination of the botanical origin of honey, consists of the pollen analysis (melissopalynological analysis), because the pollen reflects the type of vegetation from which nectars were collected. This analysis presents some limitations, since a good knowledge of the morphology of pollen and qualified staff are needed. Moreover, due to the limited amount of pollen present in the samples, usually it takes a long time to perform the analysis.

This work has as purpose to classify the honeys, both on the basis of botanical origin and in terms of geographical origin. Analyses of the phenolic fraction present in honey were conducted by HPLC-DAD. The honey samples were also analyzed by NIR spectroscopy and the classification method Partial least squares-discriminant analysis (PLS-DA) was applied to both the chromatographic and the spectroscopic data.

In particular, aim of the present project was to develop a reliable method to characterize the floral origin of honeys produced in the same geographical area (in order to avoid problems related to geographical variability), and which could constitute a valid alternative to pollen analysis. As said, at the same time, a similar approach was followed, to discriminate the geographical origin of polyfloral honey. The methods developed are easy, quick and objective, not relying on the subjective interpretation of the analyst as instead happens for the melissopalynological analysis.

HPLC-DAD analysis of the polyphenol content present in honey was chosen for instrumental fingerprinting because it is strongly linked to the geographical and floral origin of the foodstuff, and to the climatic characteristics of the local production [102-105]. The literature describes numerous analytical methods developed to test the authenticity of honey, in particular with respect to the declared geographical and floral origins [106-108]. However, a simple and effective procedure has not yet been identified which, individually, is sufficient to the evaluation of the "complex" characteristics of a honey, such as its geographical or floral origin or, more generally, its belonging to a particular denomination of origin.

The polyphenol content could be a significant "discriminating" factor in order to establish a standard method (currently absent) for the analytical control of the geographic and floral origin of honey. In fact, from the literature, phenolic compounds appear to be an important quality parameter which gives the product added value and it also justifies the different cost of sales [109]; in addition, observing the differences in the quantitative composition of phenolic compounds (i.e. the fingerprint of the whole phenolic fraction) may be more useful for the

characterization (geographical and floral origin) of this product than focusing on the content of a specific substance [110].

At the same time, a spectroscopic method based on NIR spectroscopy was also developed, as NIR spectroscopy allows analyzing the honey samples as they are without any sample preparation at the same time permitting the investigation of many samples in a limited time, with a consequent economic saving.

4.2 HONEY SAMPLES

The honey samples used in the study of the botanical origin were all from the same geographical area of production (Tuscany, Italy), to avoid that the botanical classification could be affected by the different geographical origin of the product. The botanical species considered and the number of available samples are shown in Table 4.1. As it can be seen from the table also honeydew honey samples were analyzed, produced from honeydew, obtained by the action of parasitic insects.

table 4.1: botanical origin of honey samples and number of samples analyzed by HPLC-DAD and NIR spectroscopy

Floral origin	**number of samples (2012)**
acacia (ac)	10
orange (or)	10
chestnut (ch)	10
eucalyptus (eu)	10
lavender (la)	10
honeydew (hd)	10
linden (li)	10

These 70 samples, each coming from a different manufacturer of honey, were analyzed by HPLC-DAD and NIR.

On the other hand, for the study of the geographic origin of the product, samples of wildflower honey coming from different regions and countries were used. In particular, the characteristics of the samples which were analyzed by NIR spectroscopy are reported in Table 4.2.

Table 4.2: geographical origin of honey samples and number of samples analyzed by NIR spectroscopy

geographical origin of honey (analyzed by NIRS)	**number of samples (2011)**	**number of samples (2012)**
southern Greece	10	10
northern Greece	10	10
southern Italy	10	24
center Italy	15	20
northern Italy	21	20
center France	10	24
center Denmark	10	10

Unfortunately, for some of these samples only a very limited amount of honey was available, so that it was not possible to carry out the chromatographic analysis. Therefore, HPLC-DAD characterization was performed on a sub-set of the samples reported in Table 4.2: the composition of the sample set used for the geographical classification by HPLC-DAD is described in Table 4.3.

Table 4.3: geographical origin of honey samples and number of samples analyzed by HPLC-DAD

geographical origin of honey (analyzed by HPLC-DAD)	**number of samples (2011)**	**number of samples (2012)**
Greek: south	10	10
Greek: north	10	10
Italy: south	10	10
Italy: center	10	10
Italy: north	10	10
France: center	10	10
Denmark: center	10	10

4.3 MATERIALS

4.3.1 SOLVENTS

Methanol RPE for analysis (Sigma-Aldrich, Milan)
Methanol RS for HPLC (Sigma-Aldrich, Milan)
Acetonitrile RPE for analysis (Sigma-Aldrich, Milan)
Acetonitrile RS for HPLC (Sigma-Aldrich, Milan)
Concentrated hydrochloric acid(Carlo Erba, Milan)
Milli-Q water (distilled water further purified with Milli-Q Plus apparatus).
Concentrated formic acid (Carlo Erba, Milan)

4.3.2 STANDARDS

Apigenin (Sigma-Aldrich, Milan)
Ferulic acid (Sigma-Aldrich, Milan)

p-hydroxybenzoic acid (Sigma-Aldrich, Milan)
Kaempferol (Sigma-Aldrich, Milan).

4.3.3 INSTRUMENTATION AND SOFTWARE

The extraction of the phenolic component present in honey was performed using C18 SPE cartridges (Applied Separations); the chromatographic analysis was performed using a HPLC apparatus SpectraSystem LC of ThermoFisher, equipped with a degasser SCL1000 model, a two-way pump model P4000 and a UV-Visible photodiode model UV6000LP. The chromatograph was interfaced to a personal computer for acquiring and processing the data through the interface SN4000. The acquisition of the chromatograms and the subsequent processing of the data was performed using the software ChromQuest 5.0, supplied by the manufacturer of the instrument. The column used was Agilent XDB-C18, 250 x 4.6 mm, packed with 5μm particles.
Rotary evaporator was used in the development of the method and in the preparation of the samples.
A Nicolet 6700 FT-NIR instrument (Thermo Scientific Inc., Madison, WI), equipped with a tungsten-halogen source and an InGaAs detector, was used for the acquisition of spectra in the near infrared range. The spectra were acquired at room temperature and without any further sample treatment, in transflectance mode, through the use of an integrating sphere (Thermo Scientific Inc., Madison, WI).

4.4 SAMPLE PREPARATION

As far as NIR spectroscopy is concerned, honey samples did not require any sample preparation prior to the analysis.
On the other hand, a preliminary extraction step was necessary to prepare the honey samples for the HPLC-DAD analysis. In particular, the phenolic fraction present in honey was

extracted by SPE (solid phase extraction). Solid phase extraction (SPE) was chosen since it is one of the simplest and at the same time very efficient and versatile methods of sample preparation [111]. An experimental design was used for the choice of the optimal experimental conditions, since the efficiency of the extraction procedure may depend on several factors, potentially interacting. This approach allowed carrying out the minimum number of experiments necessary for the evaluation of the effects of various factors on the response to optimize.

In particular, a fractional factorial experimental design was used for the optimization of the solid phase extraction procedure. Five variables and two levels, coded as -1 and +1 (Table 4.4) were taken into consideration.

Table 4.4: experimental variables and levels considered

experimental variables	**LEVEL -1**	**LEVEL +1**
Sample weight	5g	10g
g sample:mL HCl ratio	1/2	1/4
clean up volume	13 mL	26 mL
kind of solvent	MeOH	MeOH:CH_3CN=2:1
Solvent volume	3 mL	6 mL

The experiments described in table 4.5 were performed using a commercial polyfloral honey.

Table 4.5: Experiemental design for SPE optimization

sample weight (g)	**g sample: ml HCl ratio**	**clean up vol. (ml)**	**kind of solvent**	**volume of solvent (ml)**	**dummy variable 1**	**dummy variable 2**	**Experiment nr.**
10	1/2	26	MeOH	3	+	-	1
5	1/4	26	MeOH	3	-	+	2
10	1/2	13	MeOH:CH_3CN =2:1	3	-	+	3
10	1/4	26	MeOH:CH_3CN =2:1	6	+	+	4
5	1/2	26	MeOH:CH_3CN =2:1	6	-	-	5
5	1/2	13	MeOH	6	+	+	6
5	1/4	13	MeOH:CH_3CN =2:1	3	-	-	7
10	1/4	13	MeOH	6	-	-	8

For each extract obtained, 20μL of solution were injected in the HPLC apparatus. The mobile phase used constituted of (A) H_2O and (B) MeOH both acidified with 0.1% formic acid. The chromatography provides an initial composition of the mobile phase of 85% of A and 15% B. The gradient was set as follows (Table 4.6):

Table 4.6: chromatographic gradient used, A means H_2O and B means CH_3OH

TIME min.	0	5	10	15	20	25	50	55	60	65
A (%)	85	85	75	75	70	70	45	25	0	0
B (%)	15	15	25	25	30	30	65	75	100	100

When the 8 experiments were done, the main effects of each variable of interest were evaluated, i.e. grams of sample, ratio of the grams of samples to the volume (mL) of HCl, volume (mL) used for the washing, type and volume (mL) of solvent used for the extraction.
The responses were considered as the sum of the areas of the peaks resulting in the chromatograms of the 8 extracts injected at 3 different wavelengths (λ = 254nm, 280nm and 340nm).
To evaluate the significance of an effect at the different λ considered, a comparison was made between the effect itself and the variability associated to the dummy variables in Table 4.5, variables that do not correspond to any experimental factor, therefore allowing the assessment of the natural between samples and instrumental variation. According to these considerations, only three variables resulted to be influential for the purposes of the optimization of the solid phase extraction procedure (Table 4.7):

1. g of sample;
2. type of solvent used for the extraction;
3. volume (mL) of solvent used for the extraction.

From the calculation of the main effects, negative values were obtained in terms of both the grams of sample and of the type and volume (mL) of solvent used for extraction. Since an effect of negative sign indicates that the experimental response decreases with increasing factor, in order to maximize the total area of the peaks in the chromatograms at 3 λ (normalized for the area of the internal standard used, apigenin), the level -1 was chosen as optimal for the variables g of sample (5 g), type of solvent (MeOH) and volume (3mL) of solvent used for the extraction.
The variables *ratio g sample / HCl volume (mL)* and *clean up volume* were found to be not influential for the optimization of the extraction procedure. Accordingly, for these variables,

the level -1, i.e. a ratio g sample / HCl volume (mL) of ½ and a volume of 13mL for clean up, was selected. Indeed, the choice of level -1 has been made to reduce the cost, speed and improve the overall efficiency of the method.

The optimized extraction procedure was the following:

1. g of sample: 5;
2. ratio g sample/volume (mL) HCl: 1/2;
3. volume used for the washing: 13mL (3mL HCl + 10mL H_2O);
4. kind of solvent used for the extraction: MeOH;
5. volume of solvent used per the extraction: 3mL.

Table 4.7: Experimental design with responses and main effects of the variables of interest

NUM. EXP.	**g sample**	**ratio g sample/vol HCl**	**clean up vol.**	**kind of solvent extractant**	**Vol solv extractant**	**dummy variable 1**	**dummy variable 2**
1	+	-	+	-	-	+	-
2	-	+	+	-	-	-	+
3	+	-	-	+	-	-	+
4	+	+	+	+	+	+	+
5	-	-	+	+	+	-	-
6	-	-	-	-	+	+	+
7	-	+	-	+	-	+	-
8	+	+	-	-	+	-	-
principal coeff. 254	-2,015	-1,15	0,3575	-2,67475	-1,9435	1,6025	-1,4475
principal coeff 280	-4,6512	-3,29375	-1,8137	-6,147375	-4,912375	1,76625	-1,60375
principal coeff 340	-0,37125	-0,03875	-0,1387	-0,846625	-0,281125	0,00875	-0,09875

4.5 VALIDATION OF THE EXTRACTION PROCEDURE

Recovery is one of the parameters to be considered for the validation of an analytical method and defines the percentage of analyte that is recovered after the extraction process.

Analysis of the recovery was performed by adding the standards of *p*-hydroxybenzoic acid, ferulic acid and kaempferol to a matrix made of 20% H_20, 40% fructose and 40% glucose, the most similar to the samples to be analyzed; apigenin (the internal standard) was added after

the extraction stage and before the step of evaporation of the solvent. Recoveries are calculated by applying the following formula:

$R\ (\%) = [(A_i / A_{S.I.})_{sample}] \times 100 / [(A_i / A_{S.I.})_{ss}]$

Where:
A_i = area of the added analyte (p-hydroxybenzoic acid or ferulic acid or kaempferol)
$A_{S.I.}$ = area of internal standard (apigenin)
ss = standard solution

Table 4.9: Recoveries obtained from an average of three experiments

	p-idroxybenzoic acid	**ferulic acid**	**Kaempferolo**
RECOVERY % (RSD)	91 (6)	86 (5)	95 (4)

As shown in the table, the recoveries calculated as the average of 3 experiments appear to be high, all over 80% with RSD less than 7%. This clearly shows that the extraction method is efficient.
Another parameter for the validation of a method is the precision, or the degree of agreement between the results of a series of measurements carried out under the test conditions specified. It is measured by calculating the per cent relative standard deviation (RSD%) or coefficient of variation (CV):

$CV = (S/x) \times 100$

Where:
S = absolute standard deviation
x = the average of the results obtained

The precision of the method is considered as:

Intra-day precision (repeatability) is evaluated as the CV of mutually independent evidence obtained under the same experimental conditions, by the same operator on the same day and laboratory, with the same instruments and the same materials.

To check if extraction procedure used is repeatable, 5 analyses are performed on the same day and the area of each analyte is measured. For each compound a RSD% always less than 2% was obtained and for each extract the sum of the areas of each chromatogram also remained practically constant. This shows, therefore, that the method proposed is repeatable.

Inter-day precision (reproducibility) is evaluated as the CV of mutually independent tests obtained by the same method and the same laboratory but on different days.

To assess the reproducibility of the method applied, 5 extractive tests (one every week, for 5 weeks) were performed. Areas of each peak were calculated and the RSD% resulted to be always less than 2%.

4.6 BOTANICAL AND GEOGRAPHICAL CLASSIFICATION BY PHENOLIC FINGERPRINT

The extraction procedure, optimized and validated in terms of recovery and precision, was then applied to the extraction of the phenolic fraction present in available honey samples.

For each sample of honey the procedure previously described was applied. Successively, 20μL of each extract were injected into the HPLC-DAD system and the chromatograms were recorded at 254, 280, and 340 nm.

The chromatographic profiles of each sample were considered as fingerprint.

4.6.1 BOTANICAL CLASSIFICATION BY HPLC-DAD

The chromatographic data of 70 samples of honey analyzed with the HPLC-DAD procedure were used for the construction of classification models.

In Figures 4.1, 4.2, 4.3 the chromatograms recorded at 254.280, 340 nm are shown.

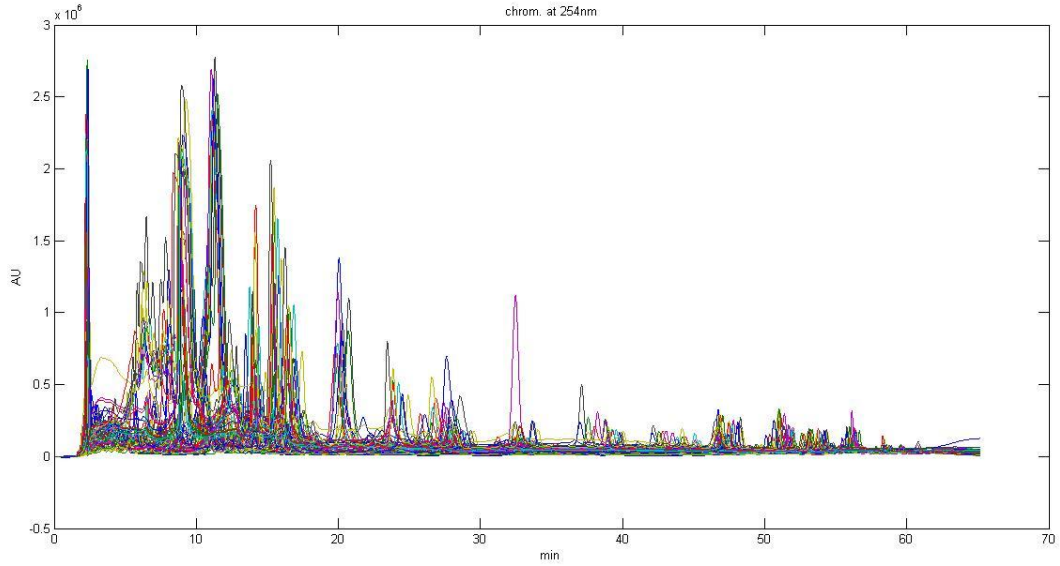

Figure 4.1: original chromatograms recorded at 254nm

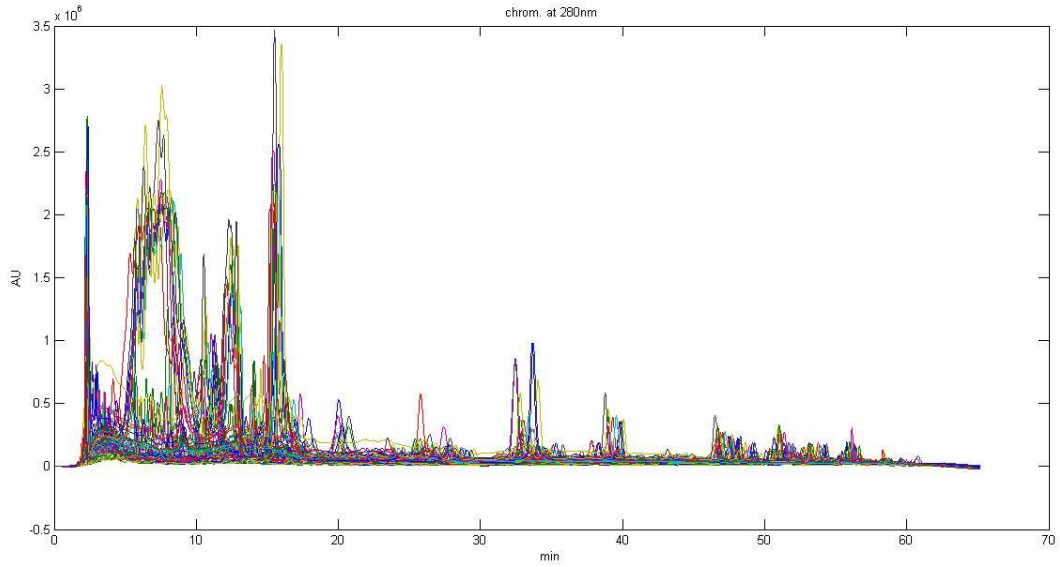

Figure 4.2: original chromatograms recorded at 280nm

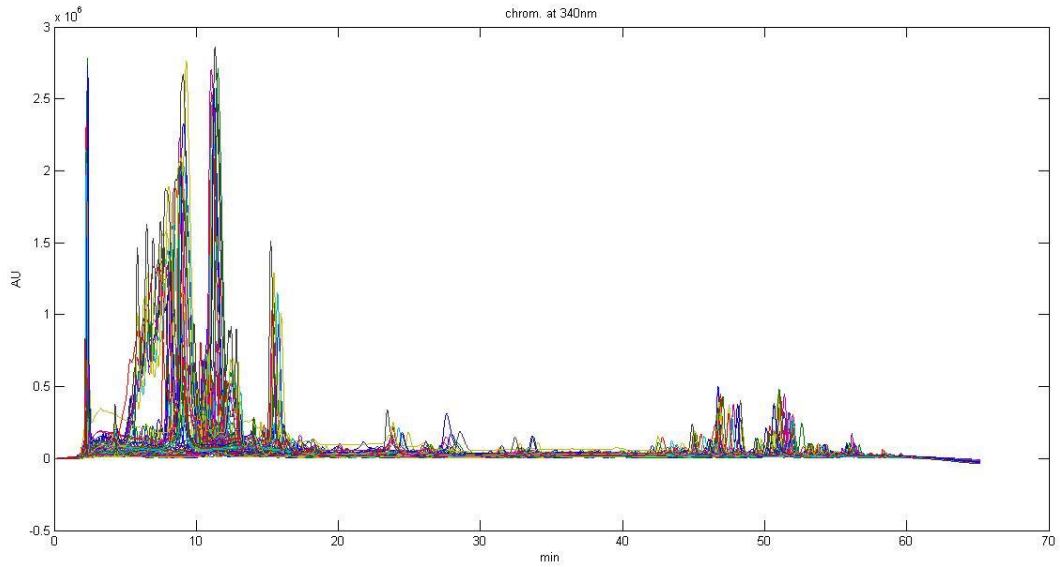

Figure 4.3: original chromatograms recorded at 340nm

Before applying the PLS-DA classification method, it was necessary to correct the baseline, to align the chromatographic peaks and, obviously, to normalize the signals, dividing them by the area of the internal standard.

Asymmetric Least Square method (described in section 2.4.1) was used to correct the baseline. The alignment of the chromatographic peaks was carried out using the algorithm Icoshift (described in section 2.4.2).

Three PLS-DA models were constructed, one for each of the wavelengths considered. The corresponding results are reported in tables 4.10, 4.11, 4.12. Considering the number of samples available for each class, the models were validated using the procedure of cross validation.

Table 4.10: PLS-DA model, data 254nm, latent variable=7, cross validation (CV)= venetian blinds w/ 10 splits

254nm	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	0.800	1.000	0.800	1.000	1.000	1.000	0.800
specificity cal.	0.950	0.983	0.950	0.983	0.983	1.000	0.967
sensitivity CV	0.600	1.000	0.800	1.000	0.800	1.000	0.800
specificity CV	0.950	0.983	0.933	0.983	0.917	1.000	0.967

Table 4.11: PLS-DA model, data 280nm, latent variable=7, cross validation (CV)= venetian blinds w/ 10 splits

280nm	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	1.000	1.000	1.000	1.000	1.000	1.000	0.900
specificity cal.	0.967	0.983	1.000	0.967	1.000	1.000	0.933
sensitivity CV	0.800	1.000	1.000	0.900	1.000	1.000	0.700
specificity CV	0.917	1.000	1.000	0.967	0.983	1.000	0.917

Table 4.12: PLS-DA model, data 340nm, latent variable=7, cross validation (CV)= venetian blinds w/ 10 splits

340nm	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	0.800	1.000	0.800	1.000	1.000	1.000	0.800
specificity cal.	0.950	0.983	0.950	0.983	0.983	1.000	0.967
sensitivity CV	0.600	1.000	0.800	1.000	0.800	1.000	0.800
specificity CV	0.950	0.983	0.933	0.983	0.917	1.000	0.967

It can be seen that the model built on the chromatographic data recorded at 280nm appears to be better than the other two. Indeed, the PLS-DA model on the chromatograms recorded at 280 nm allowed the correct classification of all the samples belonging to eucalyptus and

linden. Moreover, as far as the other categories are concerned, the lowest value of sensitivity in CV is 0.700 (only 70% of the samples of orange is correctly classified as belonging to the class orange), while the lowest value in CV specificity is 0.917.
In addition, data fusion models were built: in particular, in order to obtain more information for each sample, the following datafusion protocols were carried out:

254nm+280nm
254nm+340nm
280nm+340nm
254nm+280nm+340nm

In the following tables, the results of the models obtained on fused data are reported.

Table 4.13: PLS-DA model, datafusion of 254 and 280nm, latent variable=7, cross validation (CV)= venetian blinds w/ 10 splits

254+280nm	**acacia**	**chestnut**	**eucalyptus**	**honeydew**	**lavender**	**linden**	**orange**
sensitivity cal.	0.800	1.000	1.000	1.000	1.000	1.000	0.800
specificity cal.	0.950	0.983	0.967	0.983	0.983	1.000	0.967
sensitivity CV	0.700	1.000	0.800	1.000	1.000	1.000	0.800
specificity CV	0.950	0.983	0.967	0.983	0.983	1.000	0.967

Table 4.13: PLS-DA model, datafusion of 254 and 340nm, latent variable=6, cross validation (CV)= venetian blinds w/ 10 splits

254+340nm	**acacia**	**chestnut**	**eucalyptus**	**honeydew**	**lavender**	**linden**	**orange**
sensitivity cal.	0.800	1.000	0.900	1.000	0.900	1.000	0.900
specificity cal.	0.950	0.983	0.967	1.000	0.733	1.000	0.950
sensitivity CV	0.800	1.000	0.900	1.000	0.700	1.000	0.900
specificity CV	0.917	0.983	0.950	1.000	0.617	1.000	0.967

Table 4.14: PLS-DA model, datafusion of 280 and 340nm, latent variable=6, cross validation (CV)= venetian blinds w/ 10 splits

280+340nm	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	0.800	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.683	0.983	0.967	1.000	1.000	1.000	0.833
sensitivity CV	0.800	1.000	0.800	1.000	1.000	1.000	0.800
specificity CV	0.767	0.983	0.950	0.983	0.983	1.000	0.833

Table 4.15: PLS-DA model, datafusion of 280 and 340nm, latent variable=7, cross validation (CV)= venetian blinds w/ 10 splits

254+280+340 nm	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	0.800	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.950	0.983	0.967	0.983	1.000	1.000	0.983
sensitivity CV	0.800	1.000	1.000	1.000	1.000	1.000	0.800
specificity CV	0.950	0.983	0.967	0.983	0.933	1.000	0.983

The best model obtained in this study of classification is the one created from the fusion of the chromatographic profiles recorded at the three wavelengths. In fact, it can be seen from Table 4.15 that the model developed has sensitivity values always higher than 0.800 and specificity values of always higher than 0.933. This indicates that on average more than 80% of the samples are correctly recognized as belonging to their true class (sensitivity) and that at least 93.3% of the samples correctly rejected (specificity) as not belonging to the other categories.

For a complete interpretation of a classification model, in addition to the results in terms of percentage of correct predictions, it is also important to assess which variables of the original data set contribute the most to the classification.

In PLS-DA analysis, this interpretation can be accomplished through the inspection of the VIP scores (Variable Importance in the Projection). For each variable, in fact, the VIP

coefficient is an index of the importance of the variable in the definition of the subspace of the latent variables [112].

Operationally, since the average of the squares of VIP is 1, for the interpretation of the models all those variables for which the calculated value of the VIP is greater than this threshold are considered significant. In particular, the VIP scores corresponding to the predictions of the different categories are reported in the following figures (the interval between data points corresponds to 1s in the retention time scale, so that each chromatogram is made of 3901 points, equivalent to 65 minutes, and consequently 11703 variables = 3901 * 3 result from data fusion).

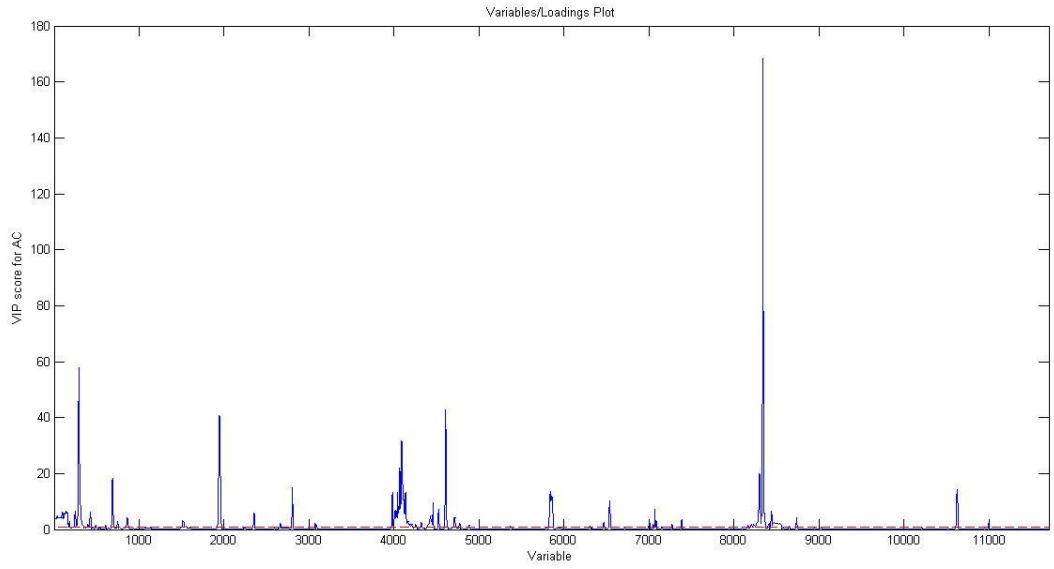

Figure 4.10: VIP score for the acacia class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points relating to the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

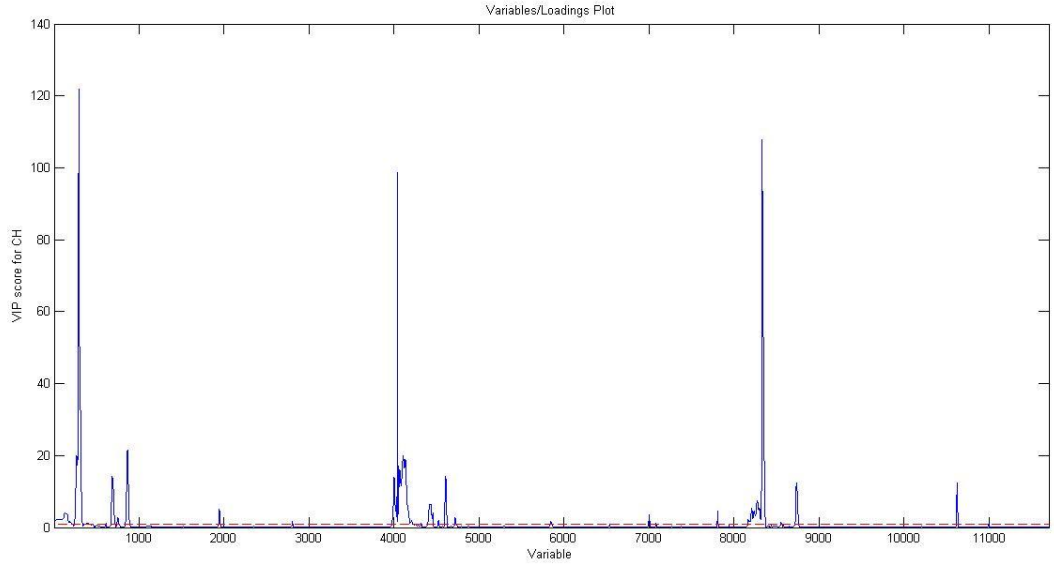

Figure 4.11: VIP score for the chestnut class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

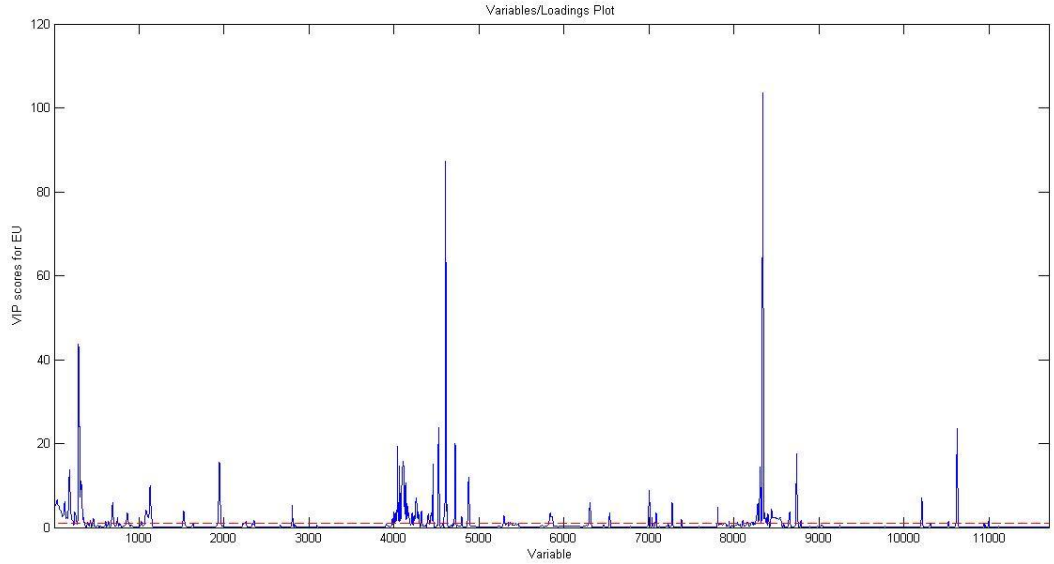

Figure 4.12: VIP score for the eucalyptus class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points relating to the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

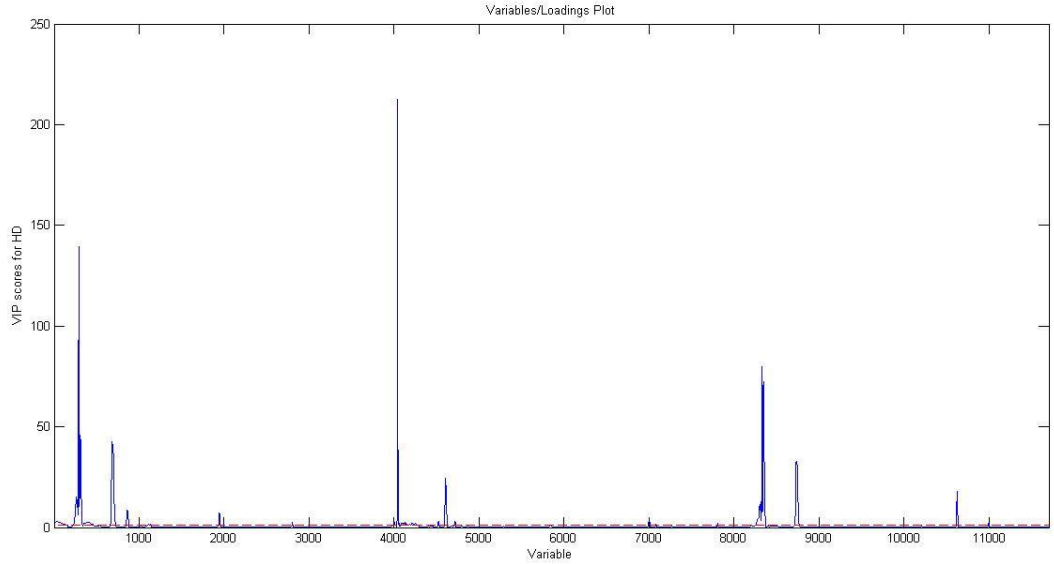

Figure 4.13: VIP score for the honeydew class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points relating to the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

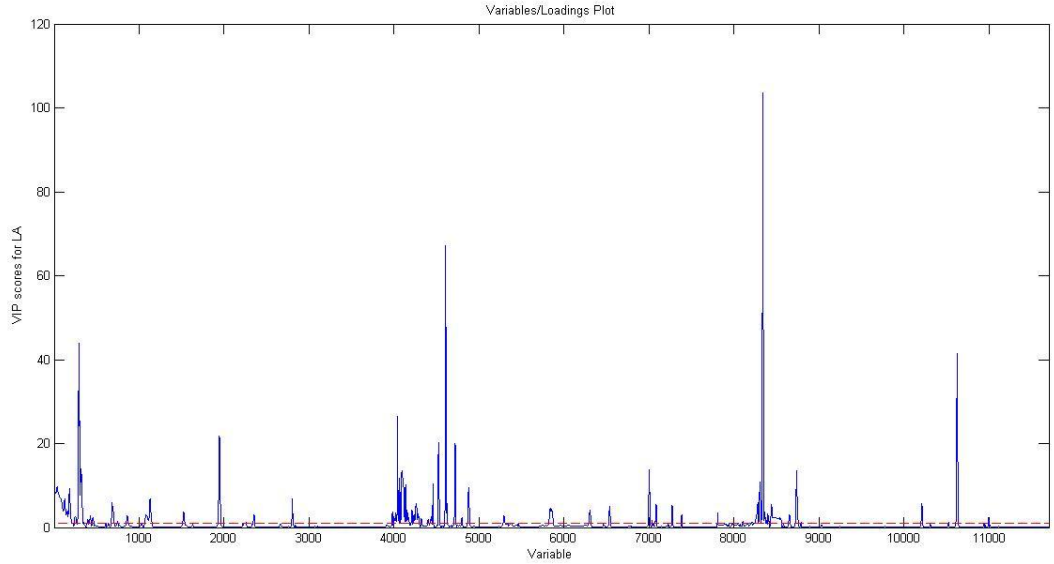

Figure 4.14: VIP score for the lavender class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points relating to the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

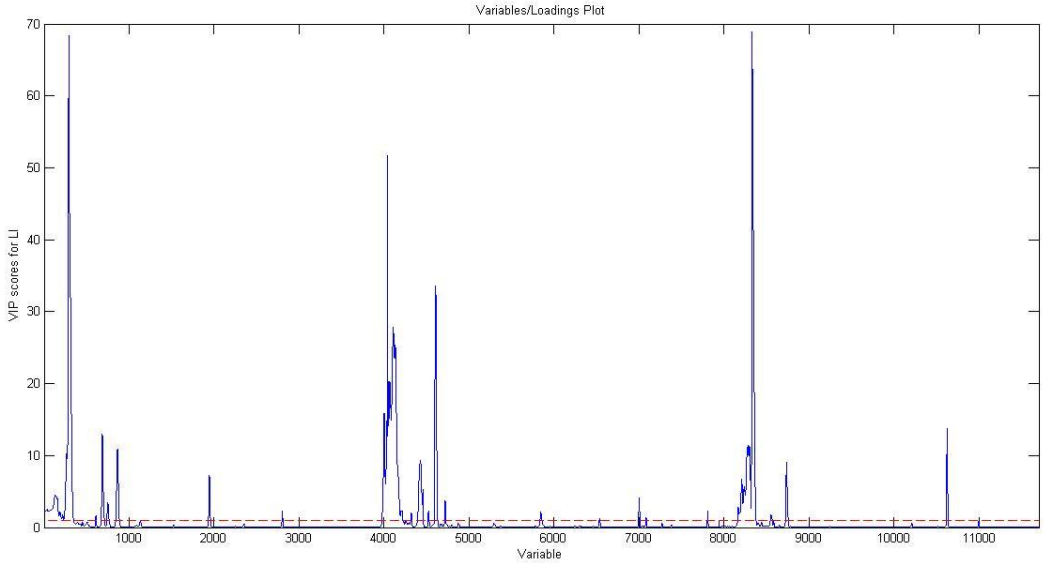

Figure 4.15: VIP score for the linden class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points relating to the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

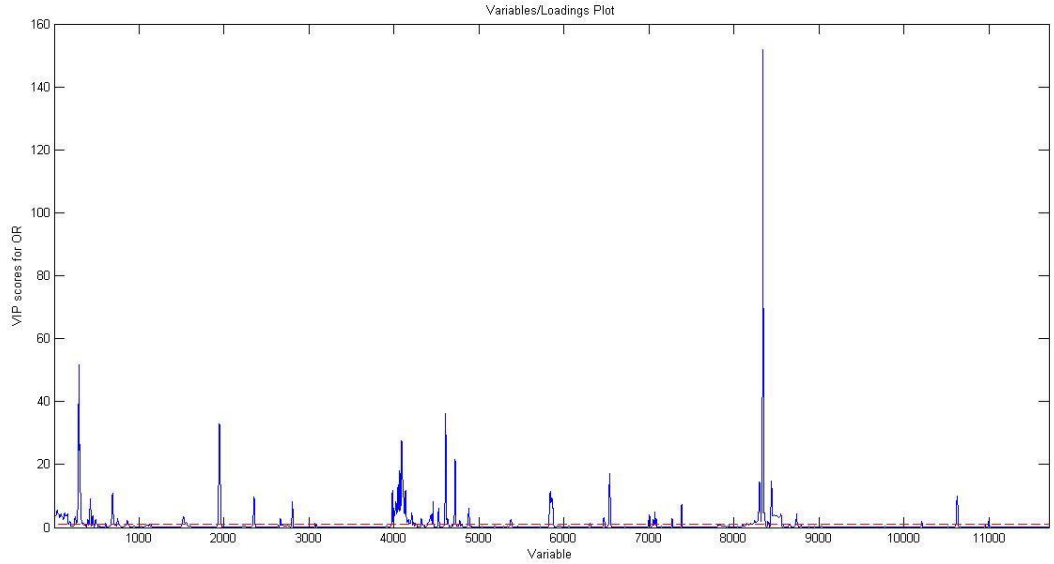

Figure 4.16: VIP score for the orange class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points relating to the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points relating to the chromatograms recorded at 280 nm; variable from 7803 to 11703 match the points regarding the chromatograms recorded at 340 nm.

4.6.2 GEOGRAPHICAL CLASSIFICATION BY HPLC-DAD

The chromatographic profiles of the 140 honey samples analyzed were used as data for the construction of classification models PLS-DA.

Before the construction of the models, pretreatments were applied to correct the baseline, and to align the chromatographic peaks and, obviously, a normalization of the chromatograms as a function of the area of the internal standard added was also made.

An example of the chromatographic data pretreatment, illustrating how the alignment of the chromatographic peaks was almost perfect, is reported in Figure 4.17.

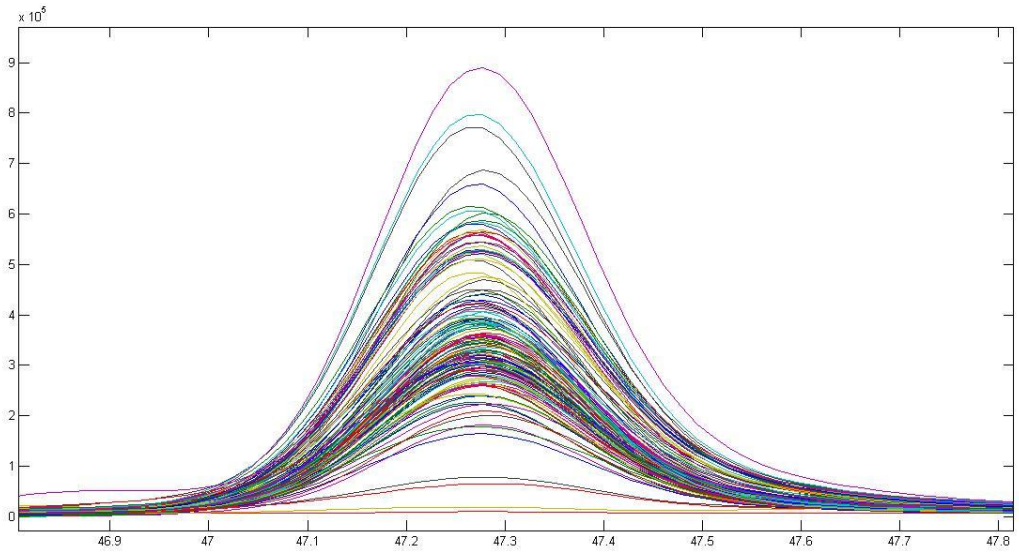

Figure 4.17: chromatographic peaks aligned after the application of the algorithm ALS; wavelength 340nm, Rt = 47.3 min.

Based on the chromatographic profiles, 3 PLS-DA classification models, one for each wavelength considered, were constructed. Specifically, the construction of the model was performed using only the chromatograms of the samples relating to the year 2011 as training set. Once the model was built and optimized, it was validated with a cross-validation procedure and also by external validation using the chromatograms of the samples produced in the year 2012 as test set. So the ability to accurately predict the geographical area of production of a honey irrespectively of the year of production was also verified.

The tables summarizing the performances of the models both in training and validation are shown below.

Table 4.16: PLS-DA model, data 254nm, latent variables=8, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

254nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	1.000	1.000	0.900	1.000	1.000	1.000	1.000
specificity cal.	0.967	0.967	0.967	1.000	1.000	1.000	0.967
sensitivity CV	0.800	1.000	0.900	1.000	1.000	0.700	1.000
specificity CV	0.967	0.900	0.900	1.000	1.000	0.900	0.933
sensitivity prediction	0.900	0.700	1.000	0.800	0.600	0.600	0.800
specificity prediction	0.950	0.933	0.817	0.983	0.933	1.000	0.883

Table 4.17: PLS-DA model, data 280nm, latent variables=7, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

280nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	0.900	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.917	0.967	0.967	0.983	1.000	1.000	0.800
sensitivity CV	0.900	1.000	1.000	1.000	0.900	0.900	0.700
specificity CV	0.917	0.950	0.950	0.900	1.000	0.967	0.850
sensitivity prediction	1.000	1.000	1.000	0.300	0.800	0.400	0.400
specificity prediction	0.917	0.883	0.800	0.967	1.000	0.933	0.750

Table 4.18: PLS-DA model, data 340nm, latent variables=3, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

340nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	0.900	0.900	1.000	1.000	0.900	0.600	1.000
specificity cal.	0.483	0.683	0.467	1.000	0.983	0.933	0.567
sensitivity CV	0.900	0.900	0.900	0.900	0.900	0.600	1.000
specificity CV	0.483	0.667	0.483	1.000	1.000	0.833	0.567
sensitivity prediction	1.000	1.000	1.000	0.900	0.400	0.500	1.000
specificity prediction	0.400	0.517	0.350	1.000	1.000	0.967	0.683

It can be seen how the model built by the chromatographic data recorded at 254nm appears to be better than the other two. The PLS-DA model on the chromatograms recorded at 254nm results in a good classification of the classes both in cross-validation and by using the external test set made up of the 2012 honeys (not used in the model construction phase). Both the values of sensitivity and specificity are good. The classes of samples that are less discriminated are northern Italy and France: in fact, the sensitivity values for these two classes is 0.600, but the value of specificity relative to the samples correctly rejected remains high.
In addition, the possibility of improving the results by fusing the data recorded at the different wavelengths was considered also in this case. In particular, the following four datafusion protocols were tested:

254nm+280nm
254nm+340nm
280nm+340nm
254nm+280nm+340nm

Table 4.19: PLS-DA model, datafusion of 254nm and 280nm, latent variables=8, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

254+280nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	1.000	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	1.000	0.967	0.917	1.000	1.000	1.000	0.950
sensitivity CV	0.900	1.000	1.000	1.000	1.000	0.800	1.000
specificity CV	0.950	0.950	0.900	1.000	1.000	0.967	0.900
sensitivity prediction	1.000	1.000	1.000	0.800	0.900	0.400	0.800
specificity prediction	0.917	0.917	0.833	0.983	0.983	1.000	0.800

Table 4.20: PLS-DA model, datafusion of 254nm and 340nm, latent variable=9, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

254+340nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	1.000	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.983	1.000	0.967	1.000	1.000	1.000	0.967
sensitivity CV	0.900	1.000	1.000	1.000	1.000	0.900	1.000
specificity CV	0.967	0.933	0.917	1.000	0.983	0.950	0.917
sensitivity prediction	0.900	0.600	1.000	0.800	0.800	0.900	0.900
specificity prediction	0.933	0.950	0.883	0.933	0.917	0.967	0.917

Table 4.21: PLS-DA model, datafusion of 280nm and 340nm, latent variables=8, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

280+340nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	0.900	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.933	0.950	0.983	1.000	1.000	0.967	0.967
sensitivity CV	0.900	1.000	1.000	0.900	0.900	0.800	0.800
specificity CV	0.917	0.933	0.950	1.000	1.000	0.933	0.967
sensitivity prediction	1.000	0.900	1.000	0.900	0.800	0.600	0.800
specificity prediction	0.867	0.967	0.867	1.000	0.950	0.917	0.967

Table 4.23: PLS-DA model, datafusion of 254nm, 280nm and 340nm, latent variables=6, cross validation (CV)= venetian blinds w/ 10 splits, prediction of test set (2012)

254+280+340 nm	Greek: south	Greek: north	Italy: south	Italy: center	Italy: north	France: center	Denmark: center
sensitivity cal.	1.000	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.983	1.000	0.983	1.000	0.983	0.983	1.000
sensitivity CV	0.900	1.000	0.900	0.900	1.000	0.900	1.000
specificity CV	0.967	0.917	0.987	1.000	0.967	0.950	1.000
sensitivity prediction	0.700	1.000	1.000	0.900	1.000	0.900	1.000
specificity prediction	0.867	0.967	1.000	0.983	0.983	0.917	0.967

The best model obtained in this classification study is the one created from the fusion of the chromatographic profiles recorded at the three wavelengths, as for the botanical classification.

In fact, it can be seen from Table 4.23 that the model correctly recognized 61 out of 70 test set samples (sensitivity), while at the same time the specificity was always higher than 86%.

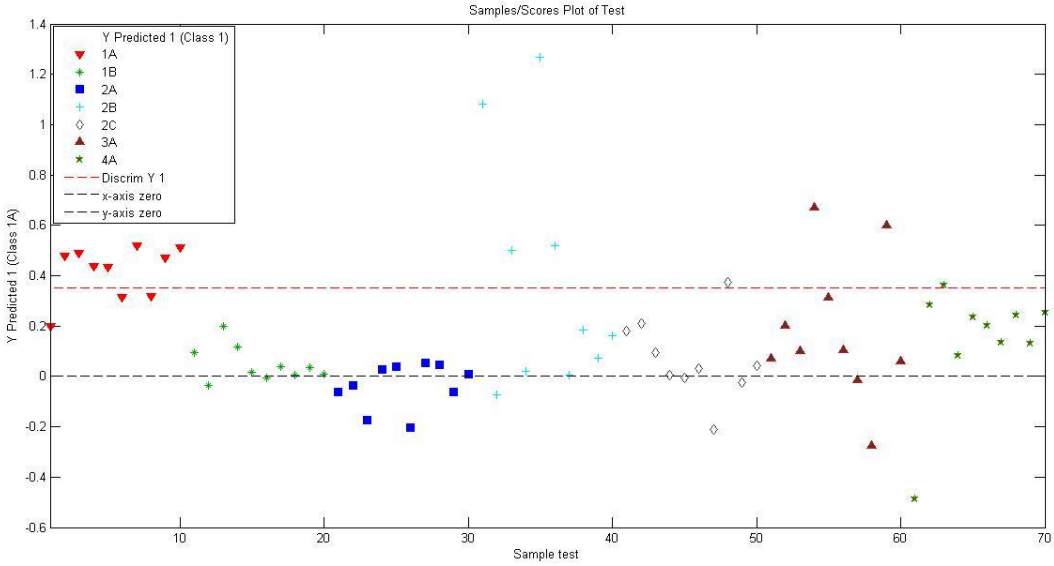

Figure 4.18: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variables=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (southern Greece)

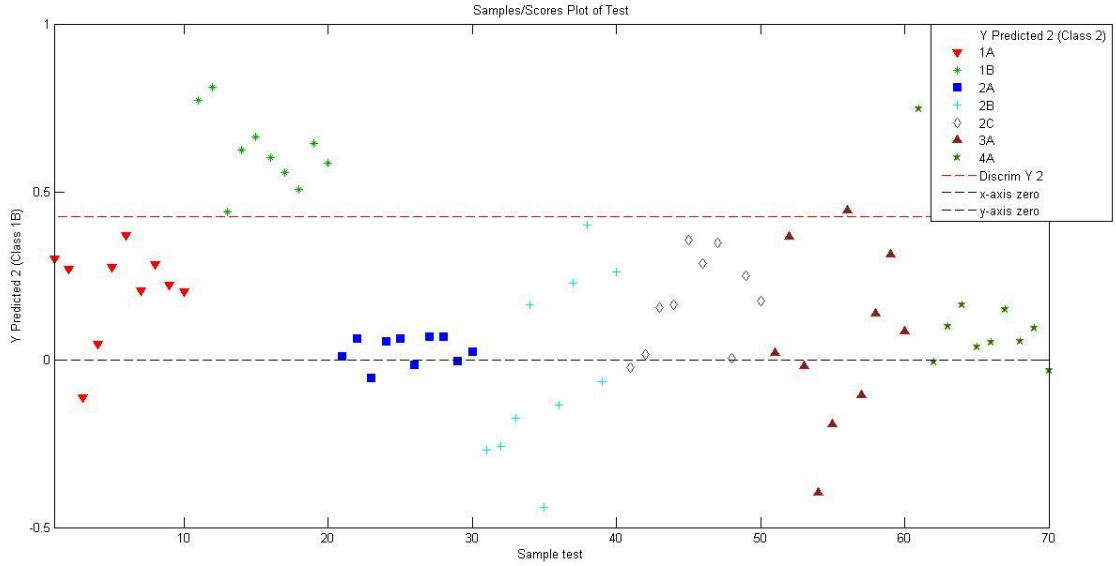

Figure 4.19: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variable=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (northern Greece)

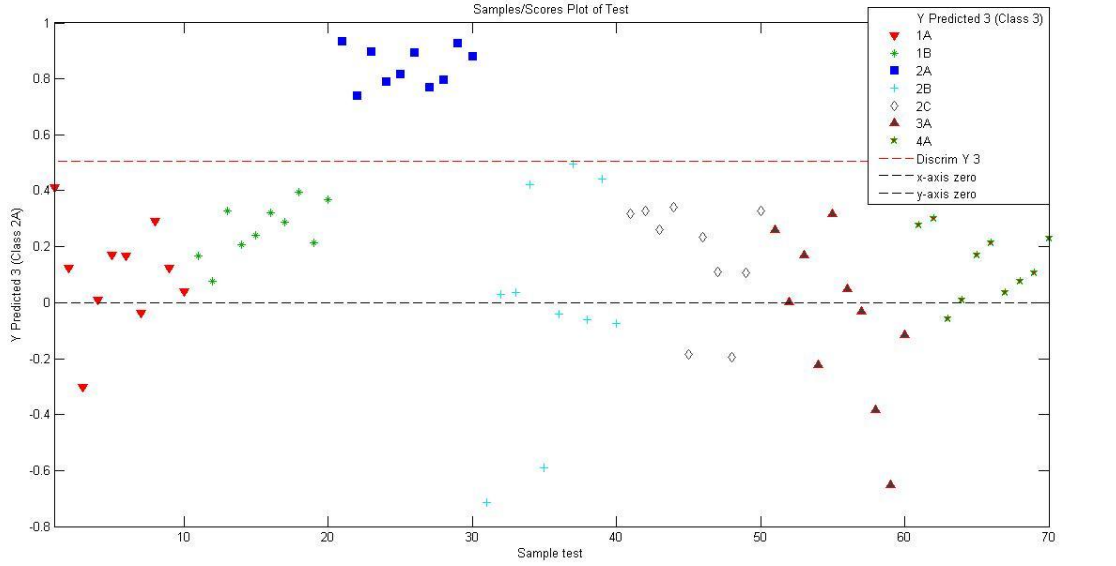

Figure 4.20: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variable=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (southern Italy)

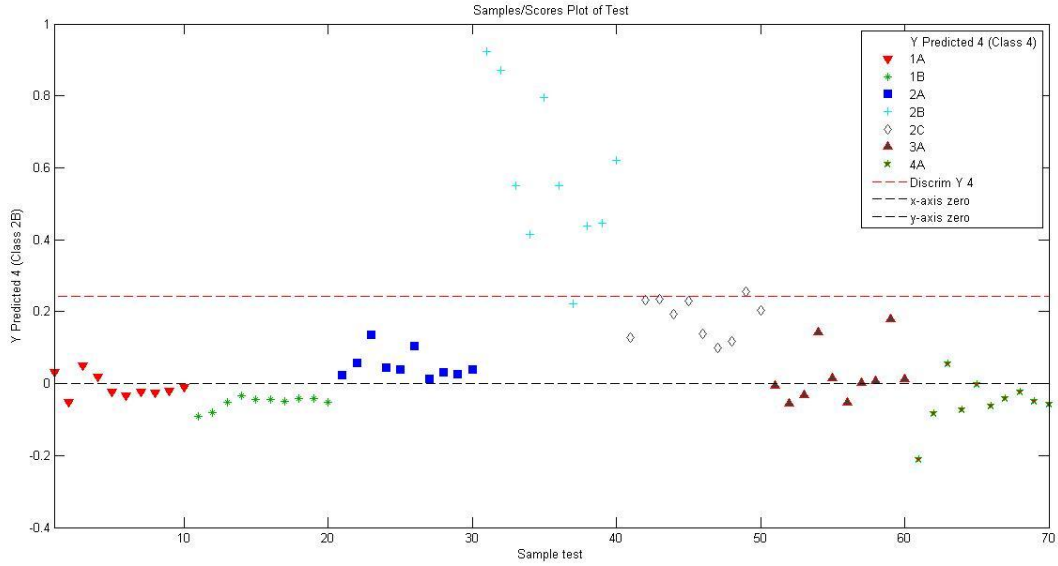

Figure 4.21: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variable=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (central Italy)

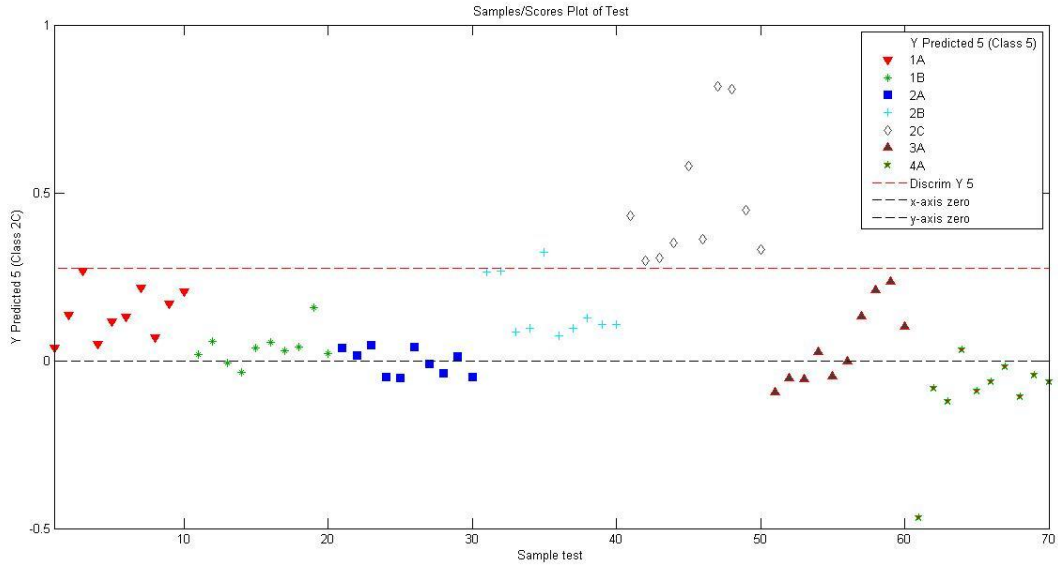

Figure 4.22: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variable=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (northern Italy)

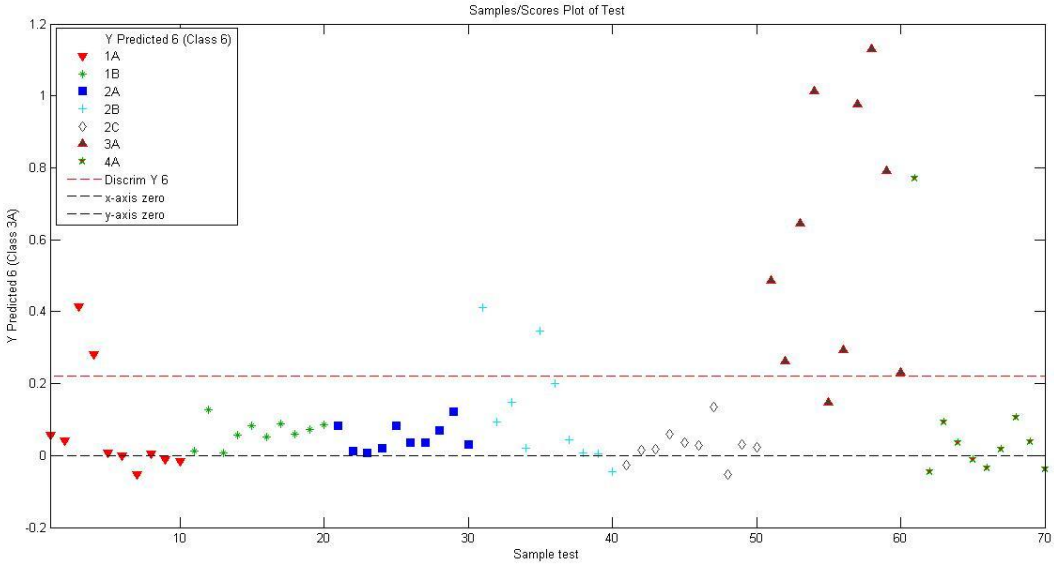

Figure 4.23: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variables=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (central France).

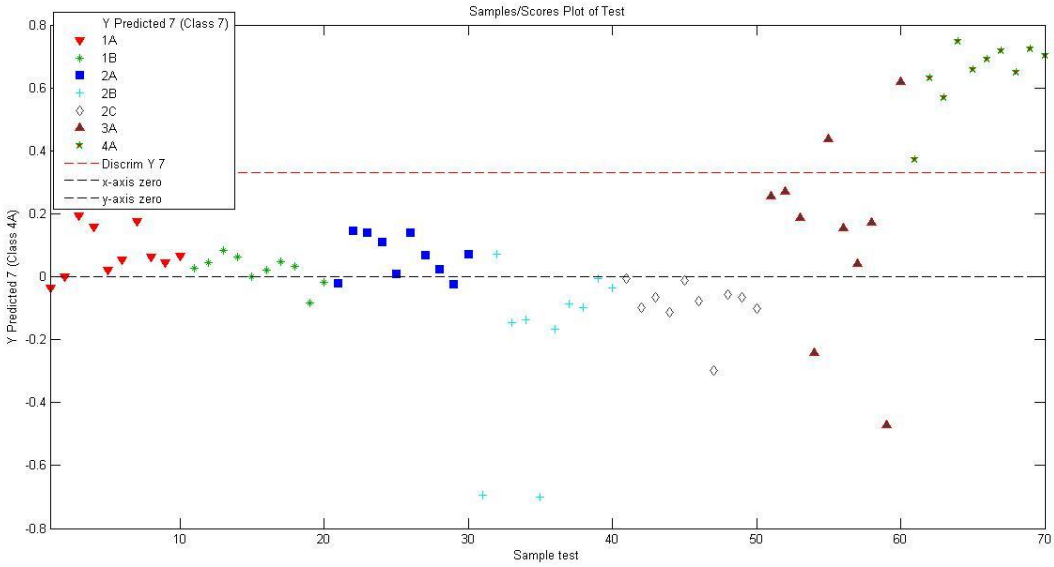

Figure 4.24: Predictive ability of the PLS-DA model, datafusion of 254nm, 280nm and 340nm; latent variables=6: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (central Denmark)

To assess which variables of the original data set contribute the most to the classification, as already described for the botanical classification, the values of the VIP (Variable Importance in the Projection) scores were inspected. In particular, the VIP scores corresponding to the predictions of the different categories are reported in the following figures (the interval between data points corresponds to 1s in the retention time scale, so that each chromatogram is made of 3901 points, equivalent to 65 minutes, and consequently 11703 variables = 3901 * 3 result from data fusion).

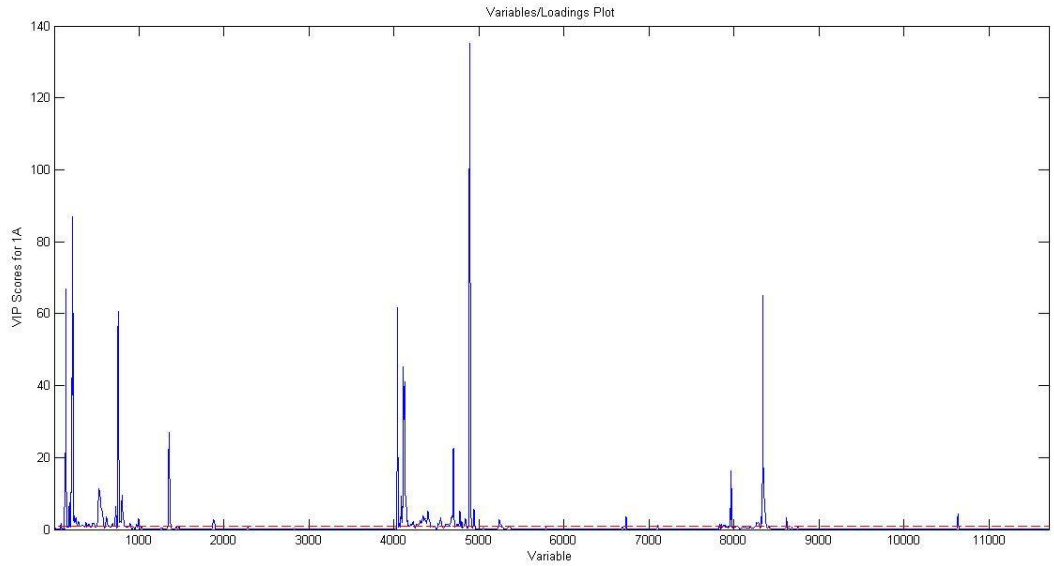

Figure 4.25: VIP score relating to the "southern Greece" class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

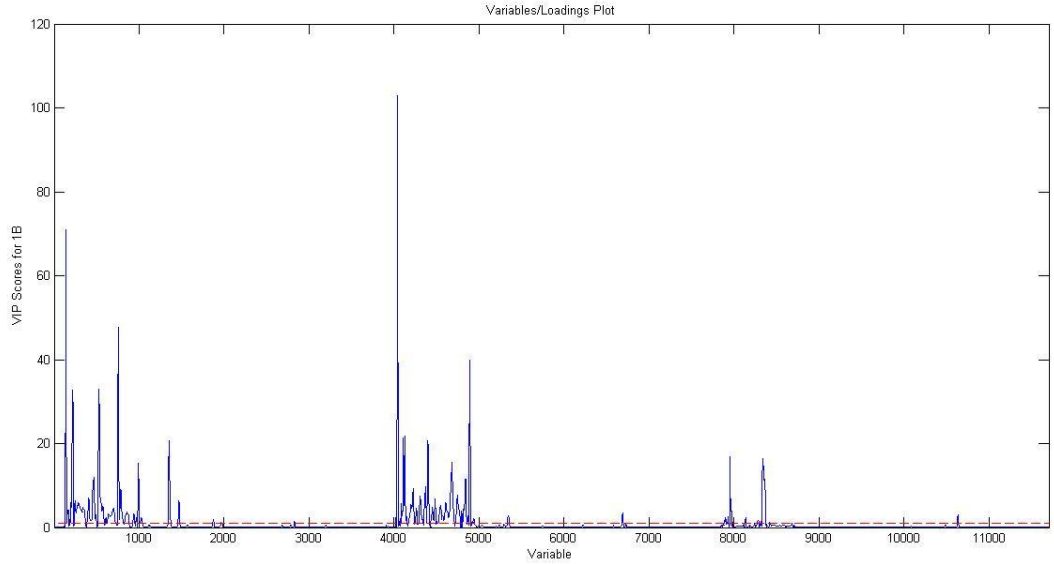

Figure 4.26: VIP score relating to the "northern Greece" class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

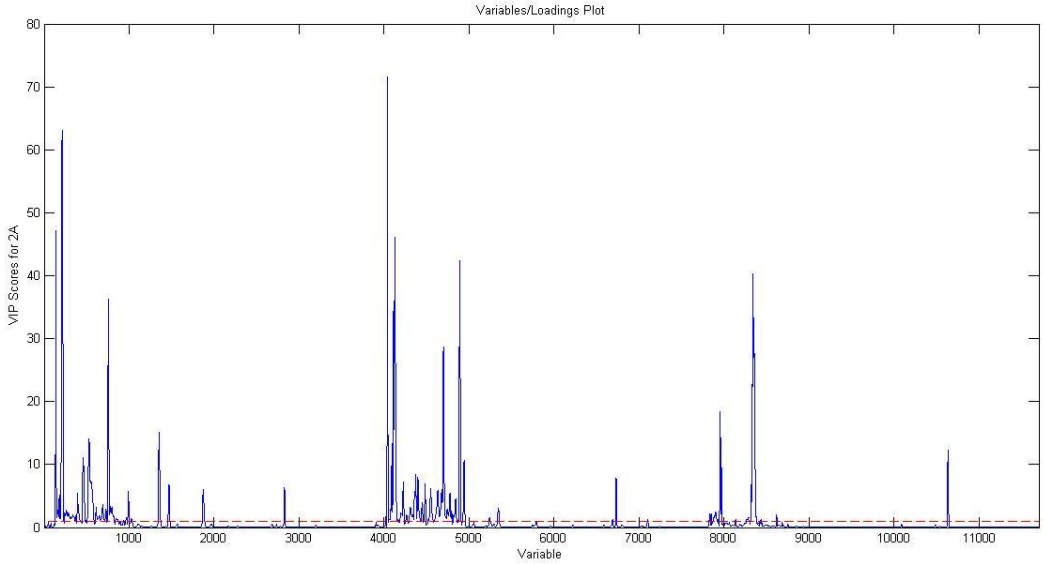

Figure 4.27: VIP score relating to the "southern Italy" class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

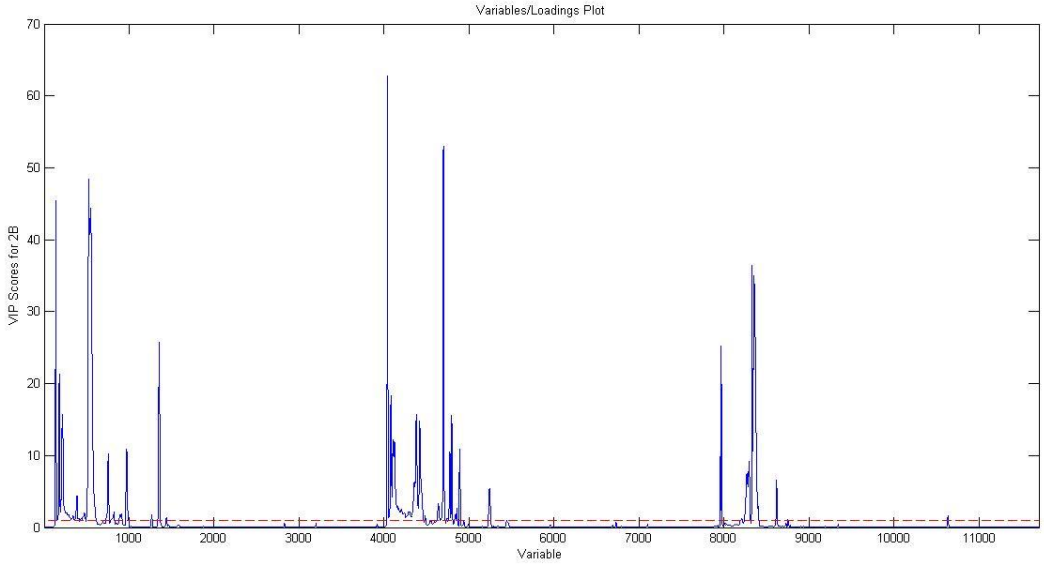

Figure 4.28: VIP score relating to the "central Italy" class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

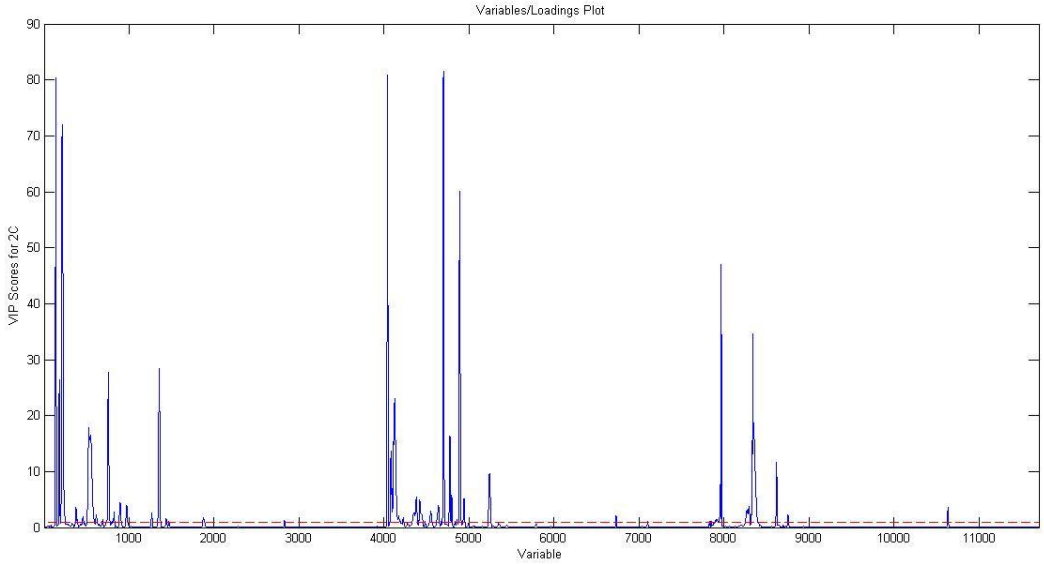

Figure 4.29: VIP score relating to the "northern Italy" class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

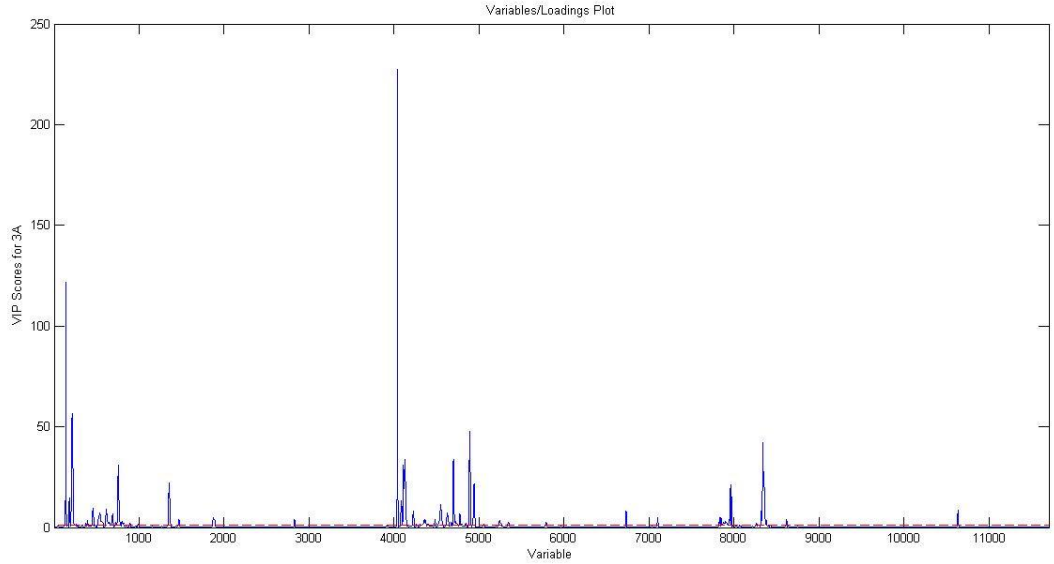

Figure 4.30: VIP score relating to the "central France" class corresponding to each chromatographic point tested; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

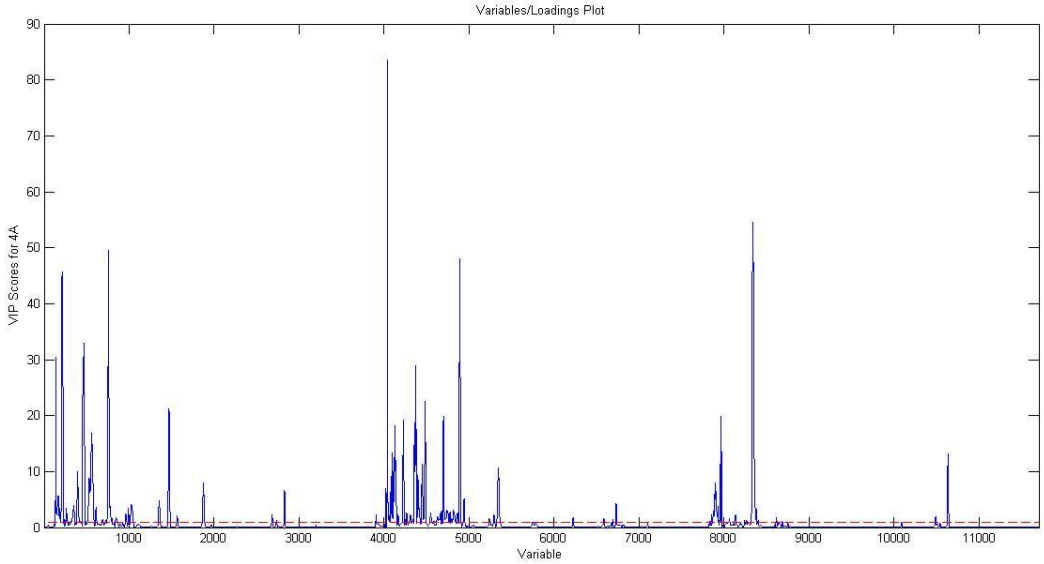

Figure 4.31: VIP score relating to the "central Denmark" class corresponding to each chromatographic point acquired; variable from 1 to 3901 correspond to the points on the chromatograms recorded at 254nm; variable from 3902 to 7802 correspond to the points on the chromatograms recorded at 280 nm; variable from 7803 to 11703 correspond to the points relating to the chromatograms recorded at 340 nm.

4.7 BOTANICAL AND GEOGRAPHICAL CLASSIFICATION BY NIR SPECTROSCOPY

As already described in section 4.1, together with the investigation of the chromatographic fingerprinting of the phenolic fraction, the possibility of discriminating the botanical and geographical origin of honeys by coupling NIR spectroscopy to chemometrics was also studied.

NIR spectroscopy is a technique which is becoming widespread in different applications of analytical chemistry, especially thanks to its desirable characteristics (in particular, the fact of being non-destructive and solvent free, requiring almost no sample treatment) [113,114]. Using this technique, through the application of chemometrics, it is possible to solve complex analytical problems.

NIR spectroscopy is an analytical technique based on the absorption of electromagnetic radiation in the near infrared region, i.e. in a range of wavenumbers between 12800 and 4000 cm^{-1}. In this interval, there are harmonics bands (overtones) and combination bands of the vibrational transitions. The spectrum obtained depends on the behavior of the sample after the incident radiation strikes: the sample can absorb, reflect or be crossed by the radiation. All this depends on the chemical and physical characteristics of the sample. The spectrum shows the intensity as a function of wavenumbers and it is characterized by broad bands, related to the functional groups that are present in the sample. The NIR spectrum can be acquired in three modalities:

- Transmittance: the fraction of the incident radiation transmitted by the sample (i.e. which is not absorbed by the sample itself) is detected.
- Reflectance: the fraction of the incident radiation reflected by the sample is recorded.
- Transflectance: this mode is a combination of the previous two. Indeed, the radiation hits the sample and passes through it; the beam transmitted by the sample is reflected back by a mirror placed in a perfectly perpendicular position with respect to it, so that the radiation passes through it again; Finally, the intensity of the resulting beam is measured.

NIR spectra, however, are very complex. A NIR spectrum alone, in fact, provides almost no quantitative or qualitative information on the sample and for this reason it is necessary to use chemometrics.

The acquisition was made through the use of a NIR spectrophotometer in transflectance mode. For each sample (previously homogenized), a drop of honey between was placed between two laboratory slides and each sample was analyzed four times, each time repeating the sampling and analysis. Once recorded through the instrumental software Omnic, the data were saved and exported to Matlab.

Successively, the NIR spectra of the 264 samples available for the botanical and geographical classification were used as data for the construction of the PLS-DA models. As already described, 4 spectra were acquired for each sample and the "standard normal variate" (SNV) algorithm was applied to preprocess the signals. Indeed, this algorithm is widely used for the removal of spurious contributions associated with scattering and/or other undesired phenomena [115]. From the practical point of view the formula for the correction used by SNV is the following:

$$X_{corr} = \frac{X_{org} - a_0}{a_1} \qquad \text{(Eq. 4.1)}$$

where:

X_{corr} is the correct spectrum; X_{org} is the original spectrum, a_0 is the mean value of the original spectrum, a_1 is the standard deviation of the spectrum of each sample.

4.7.1 BOTANICAL CLASSIFICATION BY NIR

For the botanical classification of honey, 70 samples from 7 different classes were considered. SNV algorithm was applied to the resulting 280 spectra and subsequently, the 4 measurements corresponding to each sample were averaged. To illustrate the effect of the optimal pretreatment (SNV) and of other possible preprocessing considered, the raw spectra, and the results of using SNV, alone or followed by first and second derivative, are reported in the following figures.

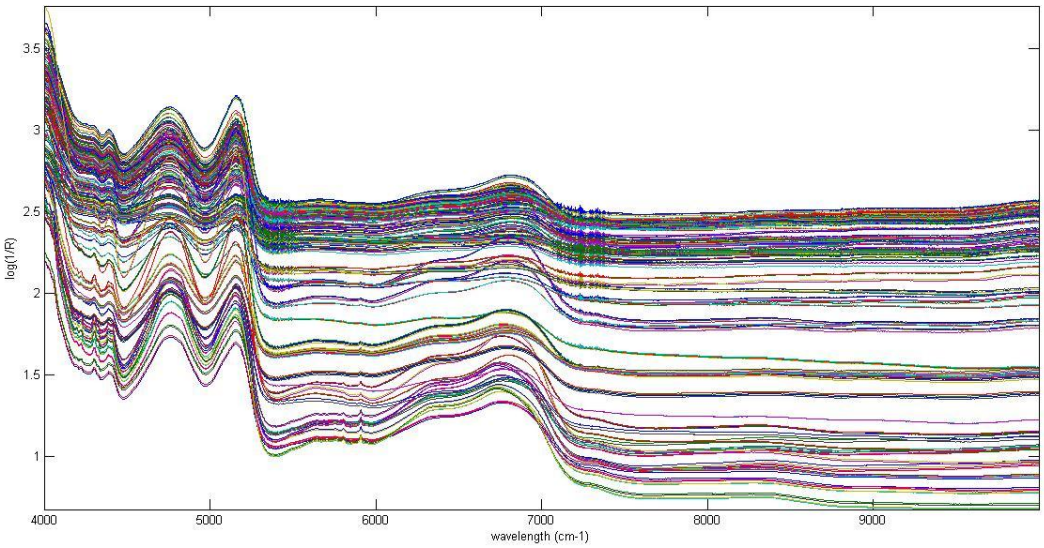

Figure 4.32: original spectra in pseudo absorbance of the 70 honey samples used for the botanical classification

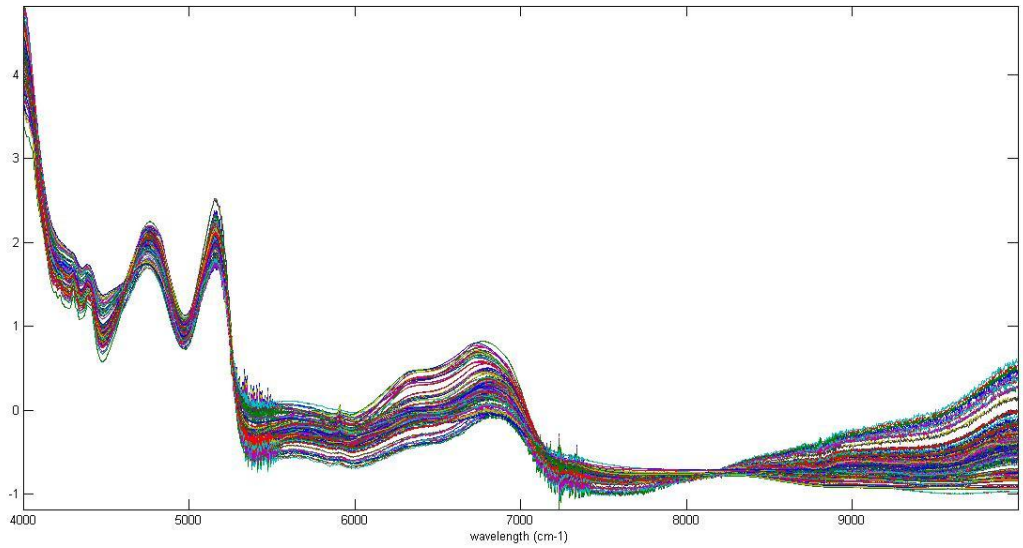

Figure 4.33: spectra pretreated with SNV

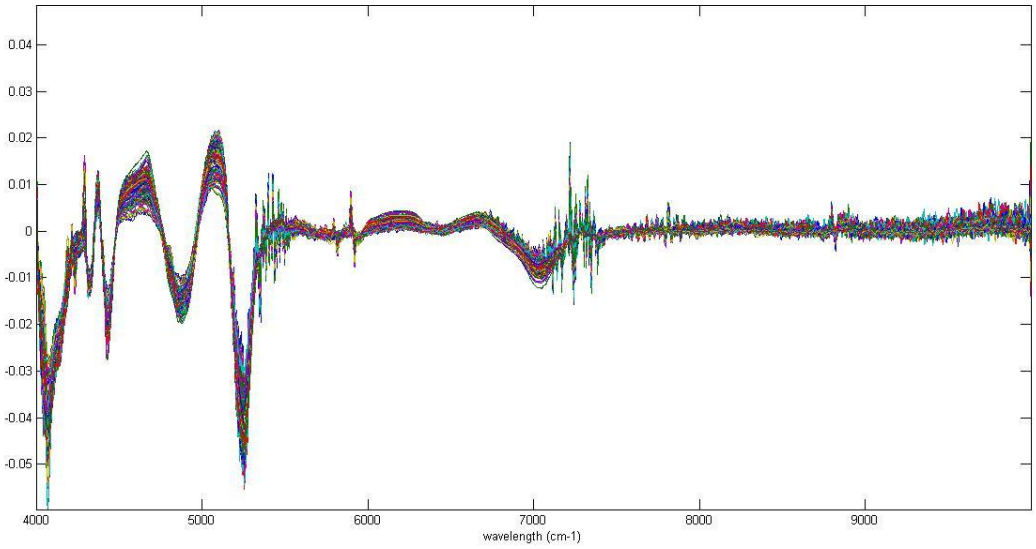

Figure 4.34: spectra pretreated with SNV +first derivative

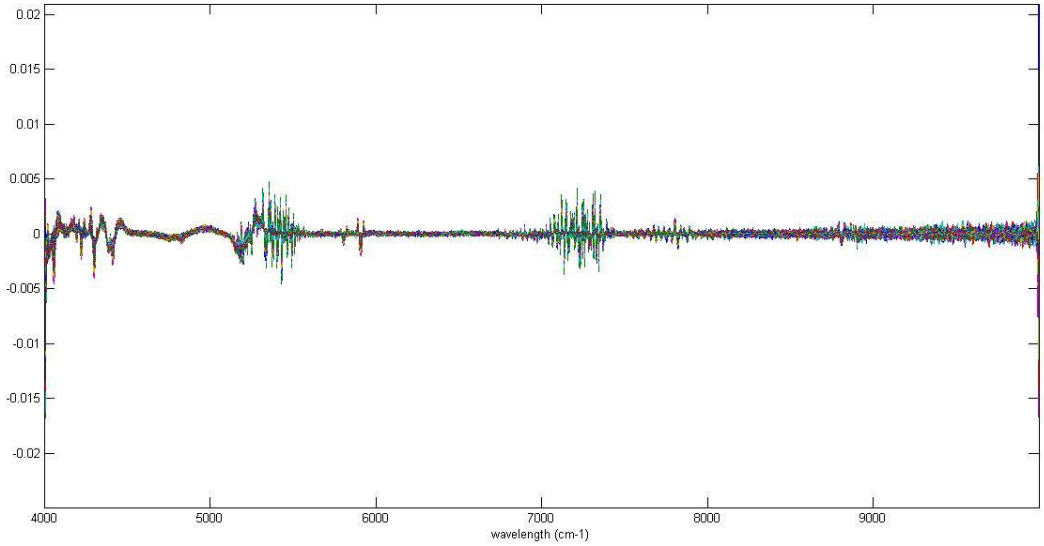

Figure 4.35: spectra pretreated with SNV + second derivative

Accordingly, three PLS-DA models were constructed (one for each type of pretreatment used). The tables below show the performances of each model (evaluated by cross-validation procedure).

Table 4.24: PLS-DA model, with SNV pretreatment, latent variable=5, cross validation (CV)= venetian blinds w/ 10 splits

SNV	**acacia**	**chestnut**	**eucalyptus**	**honeydew**	**lavender**	**linden**	**orange**
sensitivity cal.	0.900	1.000	1.000	0.800	1.000	0.900	1.000
specificity cal.	0.883	0.900	0.950	0.700	0.850	0.900	1.000
sensitivity CV	0.700	0.900	0.700	0.600	0.900	0.900	1.000
specificity CV	0.883	0.883	0.900	0.533	0.833	0.900	1.000

Table 4.25: PLS-DA model, with SNV+first derivative, latent variable=11, cross validation (CV)= venetian blinds w/ 10 splits

SNV+der1	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	0.900	1.000	1.000	1.000	1.000	1.000	1.000
specificity cal.	0.933	1.000	0.950	0.933	0.983	0.933	1.000
sensitivity CV	0.600	0.800	0.800	0.300	0.700	0.800	1.000
specificity CV	0.867	0.900	0.883	0.883	0.850	0.933	1.000

Table 4.26: PLS-DA model, with SNV+second derivative, latent variable=5, cross validation (CV)= venetian blinds w/ 10 splits

SNV+der2	acacia	chestnut	eucalyptus	honeydew	lavender	linden	orange
sensitivity cal.	0.900	0.900	0.900	0.800	0.900	0.900	1.000
specificity cal.	0.683	0.867	0.900	0.683	0.750	0.800	1.000
sensitivity CV	0.800	0.800	0.800	0.300	0.800	0.700	1.000
specificity CV	0.667	0.850	0.867	0.717	0.733	0.800	1.000

All the three PLS-DA models perfectly discriminate the orange honey, while for the other classes the best classification is obtained by SNV correction without any further differentiation. For the interpretation of the results, the VIP scores corresponding to the PLS-DA model built on the data pretreated with SNV, is reported in the following figure. The VIP plot indicates that there are different bands having a relevant impact on the construction of the model and hence on the discrimination between different botanical classes. The largest variation among the spectra of considered honey were observed in the regions corresponding to the combination bands of the C-O and C-H stretching of the saccharides between 4200 and 5200-5300 cm^{-1} and to the first overtone of O-H at 7100cm^{-1}.

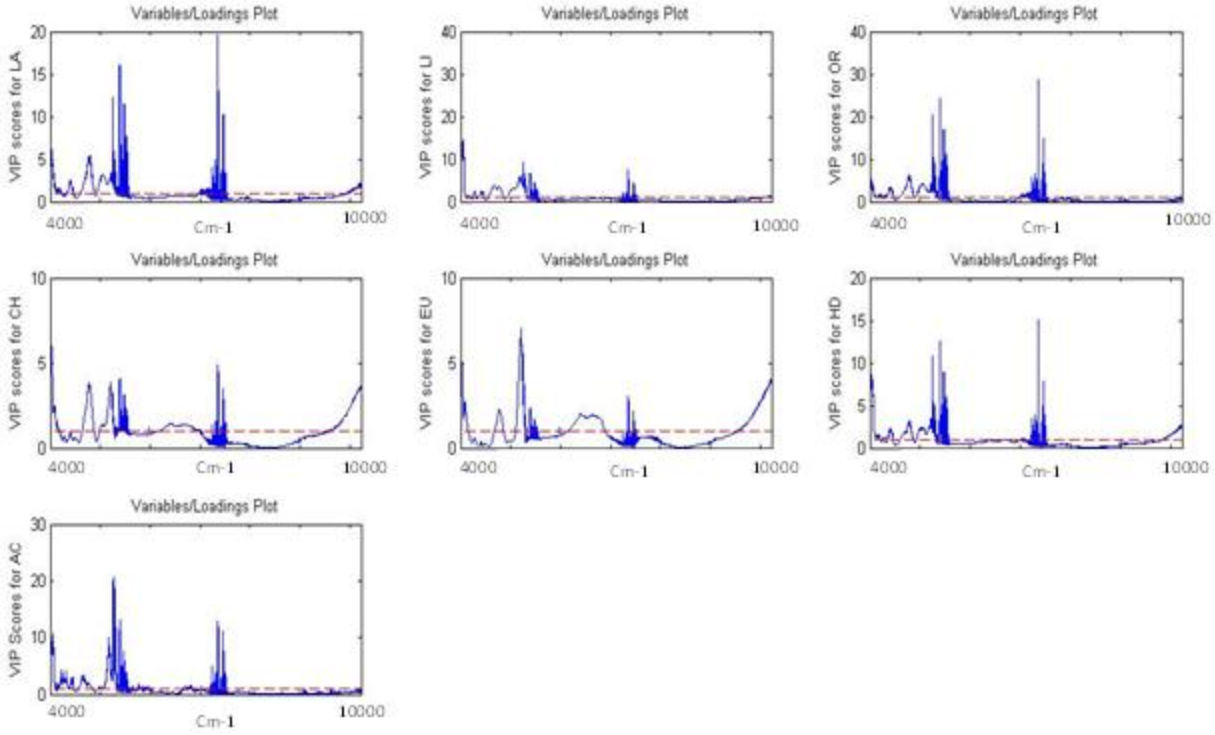

Figure 4.36: VIP score relating to the corresponding classes

4.7.2 GEOGRAPHICAL CLASSIFICATION BY NIR

204 honey samples produced in two different years of harvest were analyzed (86 samples of 2011 and 118 of 2012 samples) by NIRS to develop a method for the classification of honeys according to their geographical origin. The samples were produced by different manufacturers located in different countries and geographical areas (Table 4.2). The acquisition of the NIR spectra of each sample was performed in transflectance mode as already described in the previous paragraph. Each sample was analyzed 4 times.

As for the development of the method for the botanical classification of the honey, the NIR spectra were pretreated with SNV with the aim to remove the effects of scattering on the spectra. Subsequently the four spectra recorded for each sample, after SNV pretreatment, were averaged.

To illustrate the effect of the optimal pretreatment (SNV) and of other possible preprocessing considered, the raw spectra, and the results of using SNV, alone or followed by first and second derivative, are reported in the following figures.

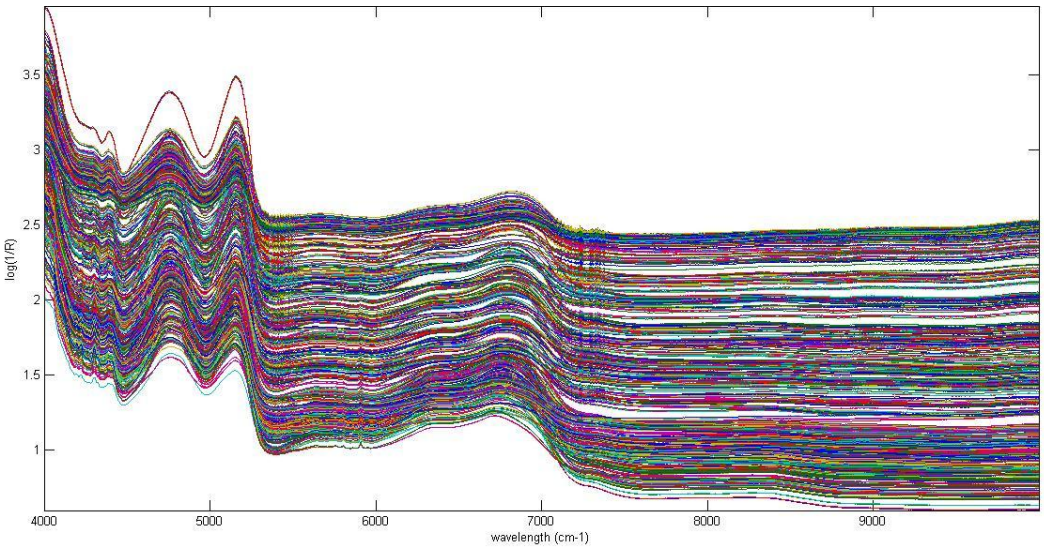

Figure 4.37: original spectra in pseudo absorbance of 204 honey samples used for the geographical classification

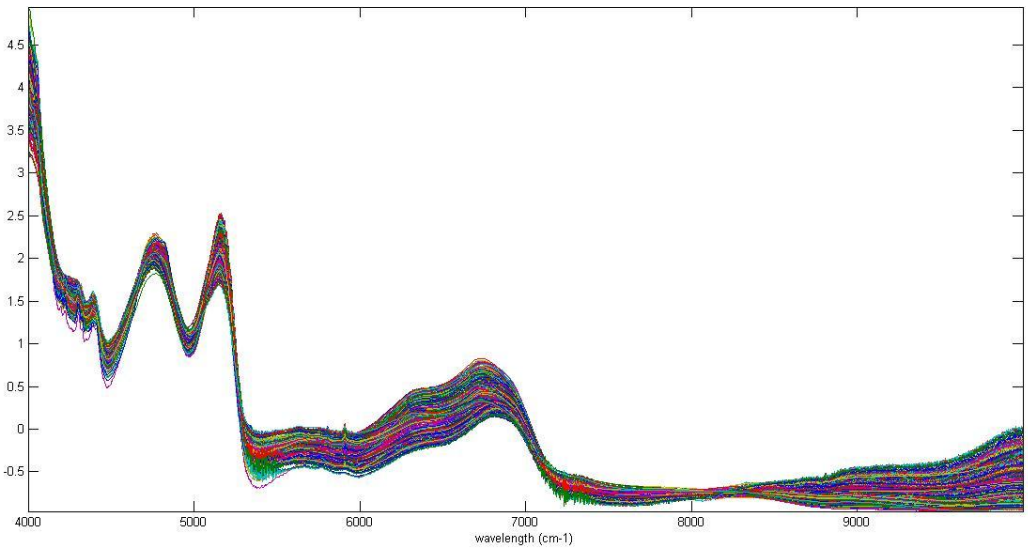

Figure 4.38: spectra pretreated with SNV

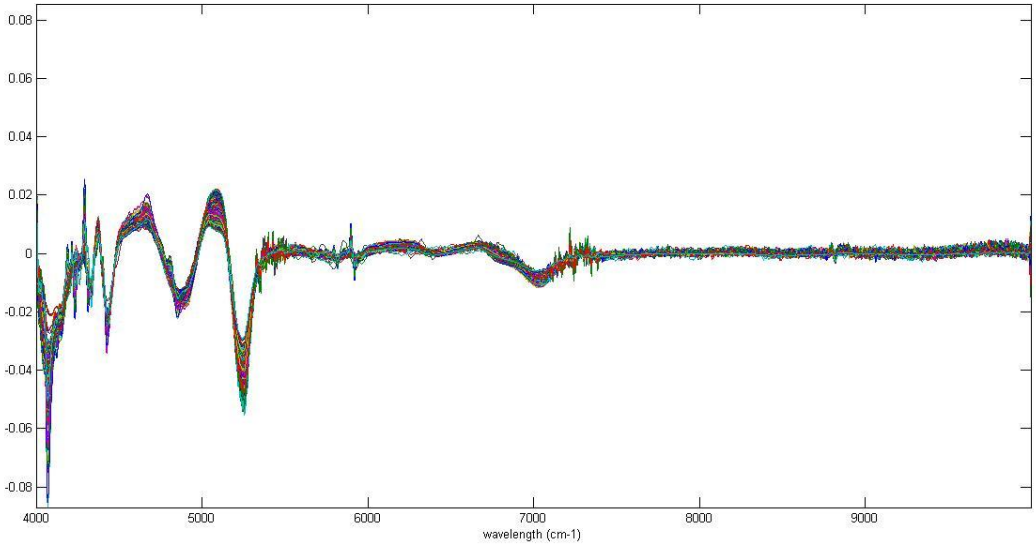

Figure 4.39: spectra pretreated with SNV + first derivative

The 204 spectral profiles associated to the different samples were at first processed by first principal component analysis (PCA) with the aim to perform exploratory data analysis; the samples of honey were plotted in the space of the first two principal components (Figure 4.40).

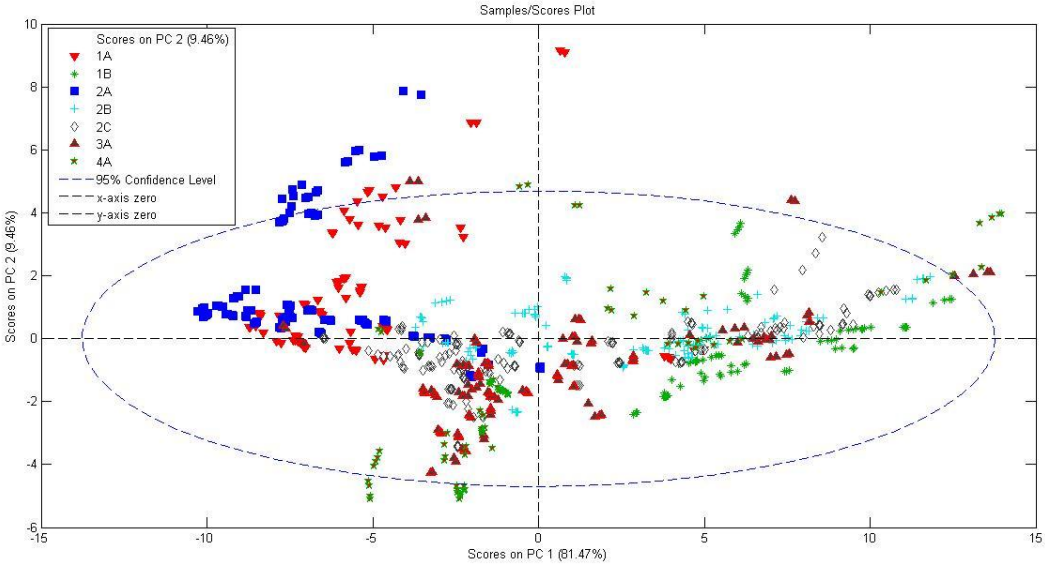

Figure 4.40: PCA scores of analyzed honey samples: 1A means southern Greece; 1B means northern Greece; 2A means southern Italy; 2B means center Italy; 2C means northern Italy; 3A means center France; 4A means center Denmark

Exploratory analysis shows the presence of evident clusters, corresponding to the different categories investigated. As done for the corresponding analysis based on HPLC-DAD, the data set was split in two sets before building the classification model: a set for the construction of the model (training set) and a set for the validation of the model built (test set). Here, it was chosen to build the model using honey samples of 2012 and to test the predictive ability of the model with samples of 2011.

Together with SNV alone, also the possibility of using first and second derivative as spectral pretreatment was tested. Three PLS-DA models were constructed, one for each type of pretreatment used (SNV, SNV + first derivative, second derivative SNV +) and the tables summarizing the performances of the corresponding models are reported below. The choice of the number of latent variables was performed based on cross validation, and each model was validated with the test set not used for the construction of the models (samples of the year 2011).

Table 4.38: PLS-DA model, with SNV pretreatment, latent variable=10, cross validation (CV)= venetian blinds w/ 10 splits

SNV	southern Greece	northern Greece	southern Italy	center Italy	northern Italy	center France	center Denmark
sensitivity cal.	0.900	1.000	0.917	0.900	0.950	0.833	0.800
specificity cal.	0.898	0.963	0.894	0.806	0.806	0.809	0.917
sensitivity CV	0.800	1.000	0.875	0.650	0.800	0.625	0.600
specificity CV	0.843	0.935	0.883	0.786	0.776	0.766	0.898
sensitivity Prediction	0.900	1.000	1.000	0.867	0.571	0.500	0.800
specificity Prediction	0.750	0.908	0.763	0.831	0.800	0.829	0.895

Table 4.39: PLS-DA model, with SNV pretreatment, latent variable=5, cross validation (CV)= venetian blinds w/ 10 splits

SNV+der1	southern Greece	northern Greece	southern Italy	center Italy	northern Italy	center France	center Denmark
sensitivity cal.	0.700	1.000	0.833	0.850	0.900	0.750	0.700
specificity cal.	0.861	0.972	0.862	0.827	0.755	0.787	0.796
sensitivity CV	0.600	0.900	0.833	0.650	0.700	0.708	0.600
specificity CV	0.852	0.963	0.869	0.847	0.745	0.723	0.806
sensitivity Prediction	0.800	0.400	1.000	0.867	0.524	0.500	1.000
specificity Prediction	0.697	0.961	0.671	0.817	0.815	0.803	0.882

Table 4.40: PLS-DA model, with SNV pretreatment, latent variable=15, cross validation (CV)= venetian blinds w/ 10 splits

SNV+der2	southern Greece	northern Greece	southern Italy	center Italy	northern Italy	center France	center Denmark
sensitivity cal.	0.800	1.000	1.000	1.000	1.000	0.917	1.000
specificity cal.	0.963	1.000	0.957	0.959	0.969	0.915	0.963
sensitivity CV	0.400	0.900	0.917	0.700	0.800	0.667	0.700
specificity CV	0.880	0.991	0.851	0.908	0.857	0.840	0.907
sensitivity Prediction	0.700	0.600	1.000	0.733	0.476	0.200	0.800
specificity Prediction	0.605	1.000	0.855	0.817	0.862	0.816	0.895

The model built from the data pretreated with SNV results to be better than the other two. The best model is able to discriminate very well the different classes, with the only exception of honeys from northern Italy and central France. In fact, excluding these two geographic classes, the sensitivity values range from 0.800 to 1000 and the specificity values from 0.750 to 0.908.

In the next figures, the predictive performances of the PLS-DA model built from the NIR spectral profiles after SNV are shown graphically.

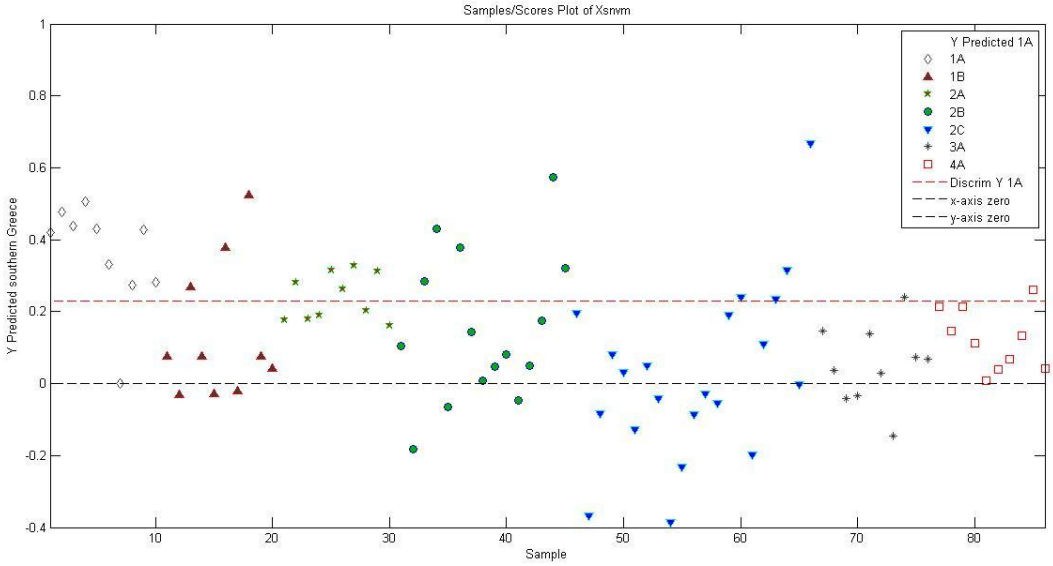

Figure 4.41: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (southern Greece)

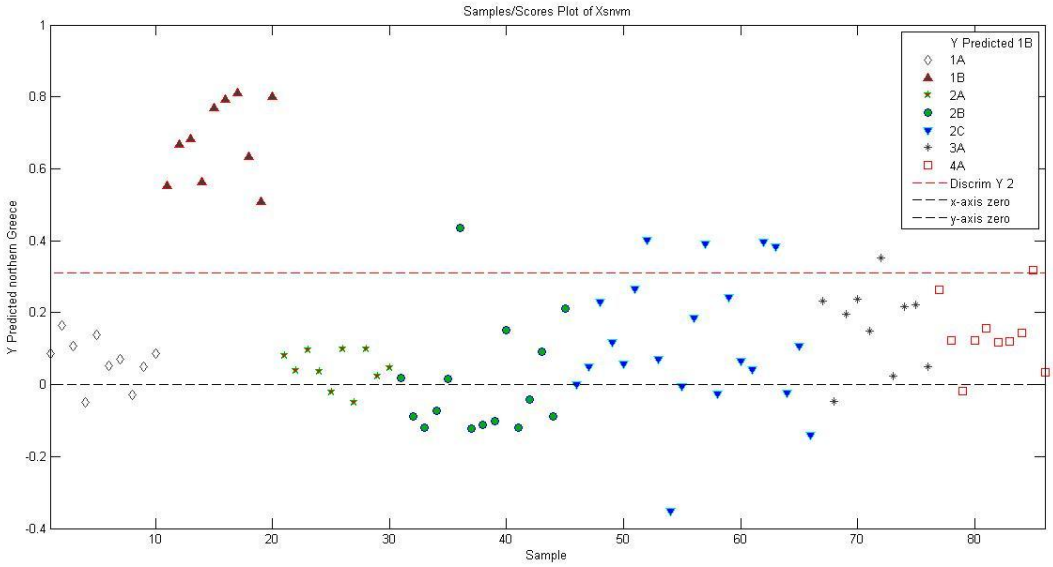

Figure 4.42: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (northern Greece).

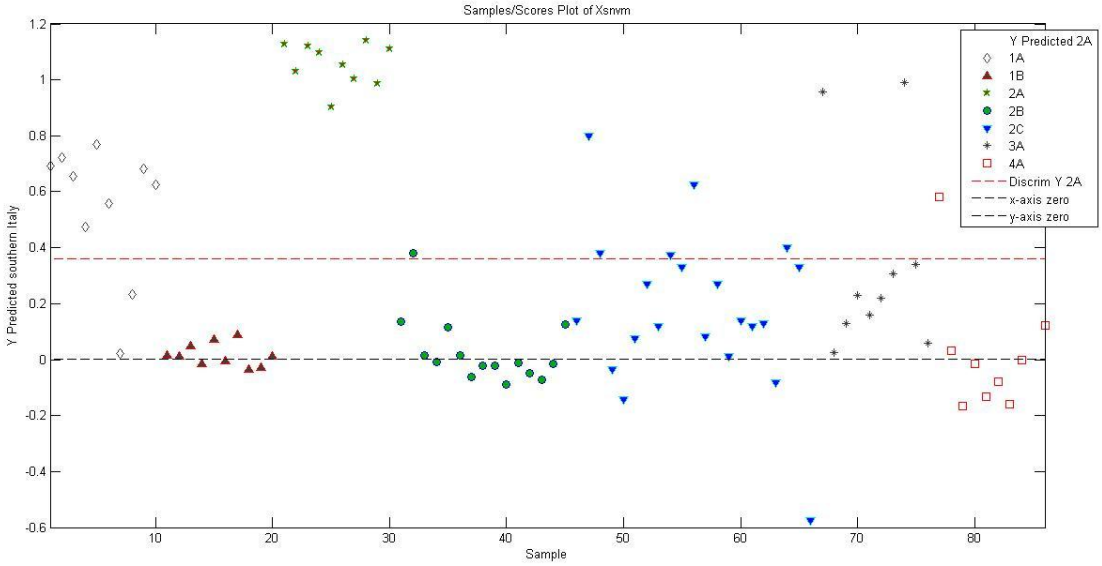

Figure 4.43: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (southern Italy).

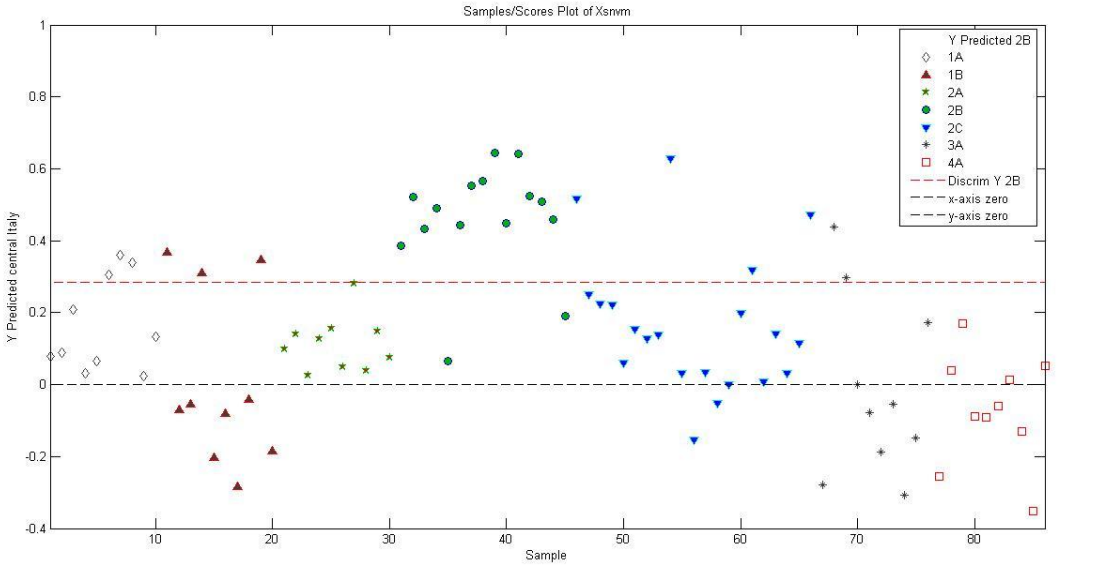

Figure 4.44: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (central Italy)

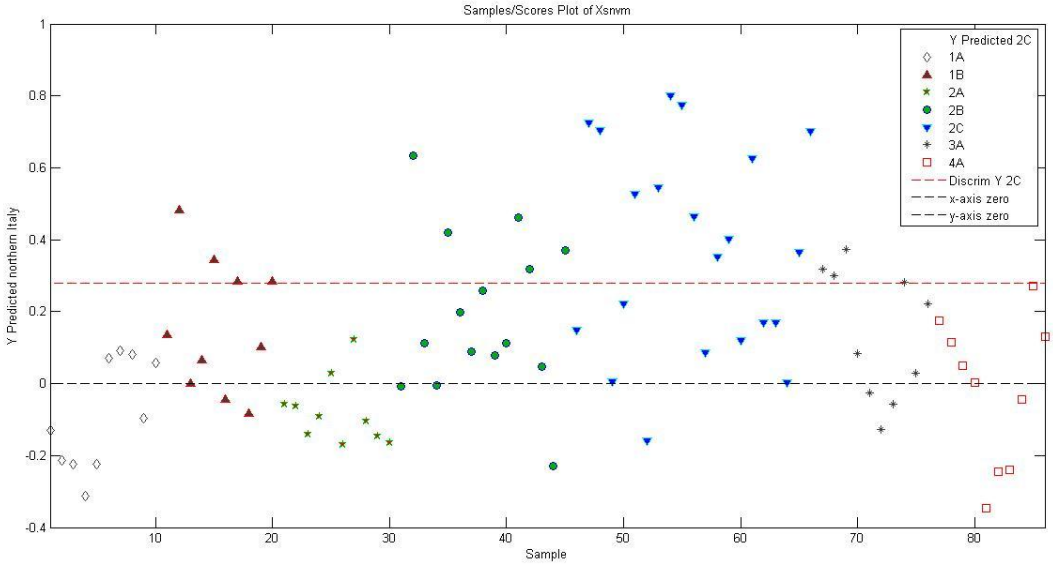

Figure 4.45: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (northern Italy)

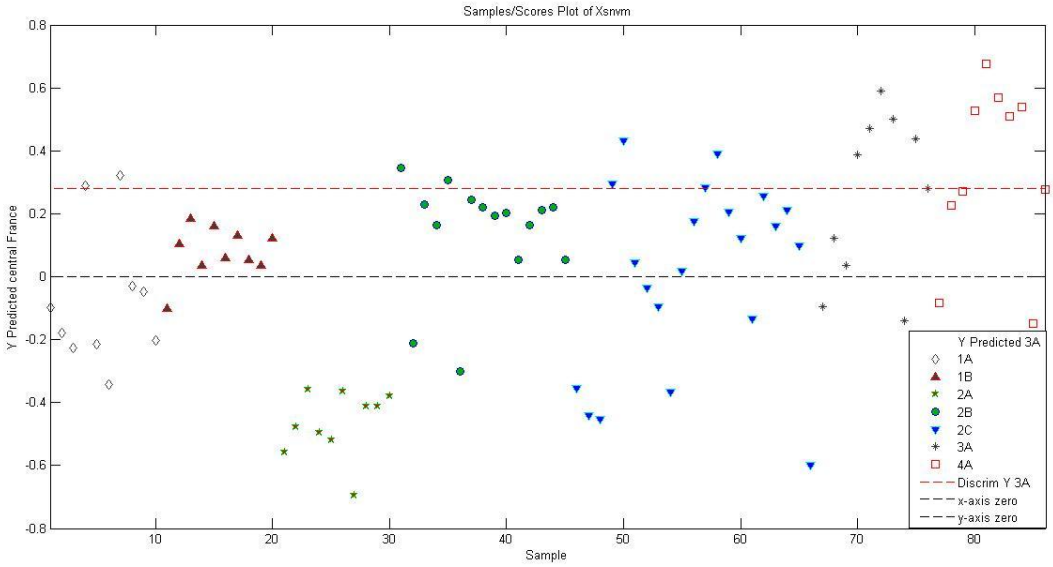

Figure 4.46: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (central France)

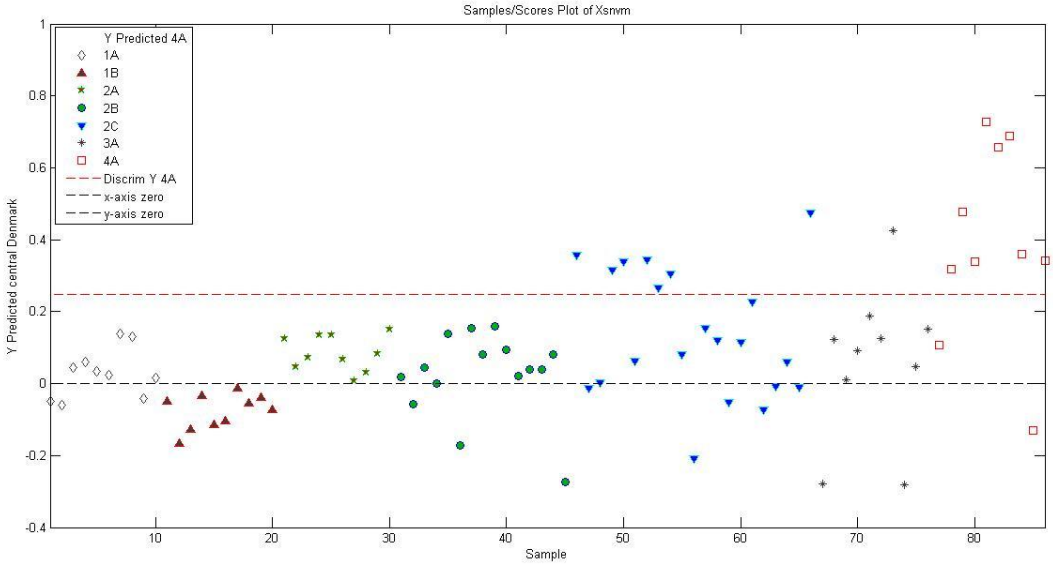

Figure 4.47: Predictive ability of the PLS-DA model; NIR data with SNV pretreatment; latent variable=10: the dashed line corresponds to the limit of the value of the predicted response over which the sample is assigned to the reference class (central Denmark)

For the sake of interpretation, the VIP scores corresponding to the model built from the data pretreated with SNV, are graphically reported in the following figure.

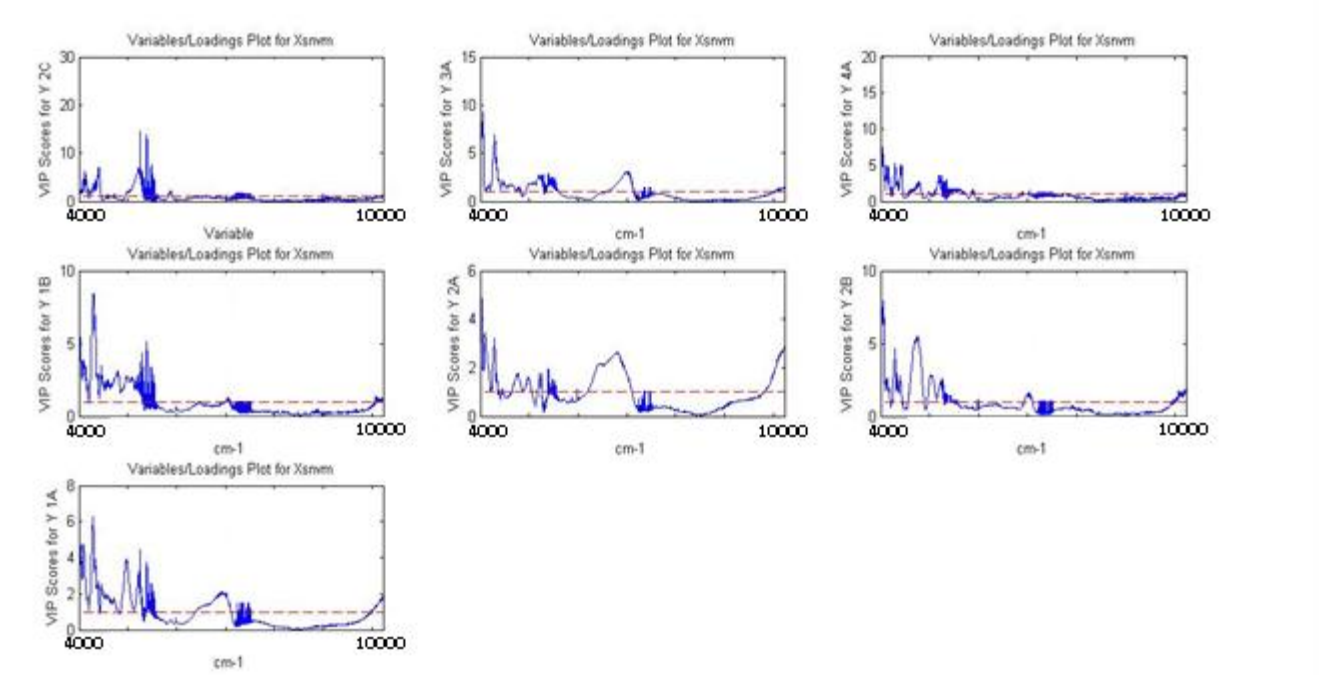

Figure 4.48: VIP score relative to geographical classes

The VIP graphs indicate that there are different bands with a significant impact on the construction of the model and therefore on the geographical discrimination between the different classes. The largest variation among the spectra of the considered honeys were observed between 4000 and 5200 cm^{-1} (combination bands of CO and CH stretching vibration and combination bands of the peptide bond) and 6900 cm^{-1} corresponding to the first overtone of OH stretching.

4.8 CONCLUSION: BOTANICAL AND GEOGRAPHICAL ORIGIN OF HONEY

The statistical processing of the NIR data and the processing of chromatographic data related to the phenolic content of all samples analyzed, led to distinguish the botanical and geographical origin of the honeys.

The HPLC-DAD and NIR protocols combined with multivariate statistical analysis, result a valuable tool for the characterization of botanical and geographical origin of honey samples.

The phenolic fraction present in honey is strongly influenced by floral origin and but also from the production area. So even honeys belonging to the same botanical species produced in different geographical regions have differences in level of phenolic composition that make them different from one other. The phenolic content represents a kind of map for the recognition of the history of the food in question, including the possibility of tracing its geographical origin.

For wildflower honey, a differentiation between Italian, Greek, French and Danish samples was obtained; the statistical models showed very good predictive abilities, being able to evaluate the geographic origin of external samples (test set).

The results of the project are promising and could be transferred to the honey production chain, for producers and processors in order to control their products; consumers will benefit from the project in terms of food safety. Finally, the method can be proposed at the control authorities for the suppression of fraud.

CHAPTER 5

HONEY: DETERMINATION OF QUALITY PARAMETERS

5.1 INTRODUCTION

One of the aims of the PhD research summarized in this dissertation was to develop an analytical approach, alternative to the official methods of analysis, based on coupling near-infrared spectroscopy with chemometric processing of the signal for the determination of some quality parameters of honey samples, so that it can be rapid, inexpensive and non-destructive/not invasive. In particular, three of the quality indices whose measured is required by law, as described in the following paragraphs, were considered: the determination of the content of reducing sugars, which usually involves the titration of Fehling's solution, the determination of water content which normally consists of a refractometric analysis, and the quantification of the 5-hydroxymethylfurfural which, as far as now, is accomplished through the use of a procedure based on liquid chromatography. At the same time, again in order to reduce the time and cost of analysis, additional tests were carried out to verify if it was also possible to determine the water content by thermo-gravimetric analysis, which is the method required by law for determining ashes, another index of honey quality. In all cases, since the development of the NIR method required to have samples for which the values of the properties to be calibrated were known and no certified standards were available, in the first

phase of the study a proper number of honey samples was analyzed by the official methods to obtain the reference values to be used for PLS modelling. Indeed, the relation between the NIR spectra and the properties whose values had to be predicted was sought by processing the data using chemometric regression methods.

5.1.1 REGULATIONS OF HONEY QUALITY

Aiming at consumer protection and fighting against frauds, the authentication of honey samples passes through an European legislation (Reg. CEE 753/82 art. 2, Official Gazette. L 86/53 del 31-03-1982) which defines the quality indices of the product. In particular, the law establishes the characteristics of honey to be sold as it is or to be used for industrial purposes, by indicating a series of parameters to be measured and their corresponding threshold values; in addition to these limits, the regulations lay also down the methods for their determination.

Table 5.1: quality criteria for honey with their respective features

reducing sugar content	not less than 65% (honeydew honey 60%); it serves to identify whether honey were added other sugars such as sucrose, which is not a reducing one.
apparent water content	not more than 21% (heather honey and strawberry clover not more than 23%); it serves to prevent the fermentation
sucrose content	not more than 5% (honeydew honey 10%); also this analysis is used to determine the addition of sucrose or other not reducing sugars
content of minerals (ash)	not more than 0,6% (honeydew honey 1%); it serves to identify contamination by mineral particles, addition of molasses or inverted sugars or sugar syrups that contain minerals
content of water-insoluble substances	not more than 0,1% (pressed honey not more than 1%); it serves to locate honeys rich in impurities
acidity	not more than 40 meq / kg; it serves to identify whether a honey is old because the acidity is a parameter that increases with aging and sophistication with other sugars or sugar syrups.
diastatic index	not less than 8(shade's scale); it serves to identify adulteration with substances that do not give diastase, excessive heating, prolonged storage at elevated temperatures
hydroxymethylfurfural content (HMF)	not more than 40 mg / kg; it serves to identify addition of inverted sugar, excessive heating and prolonged storage at elevated temperatures.

5.2 OFFICIAL METHODS

As previously mentioned, for the determination of each of these parameters, the law provides official methods, which were selected according to the required analytical accuracy and based on their characteristics which could allow to have large application in a wide number of laboratories (Ministry of Health, decree of 20 July 1984, "Methods of analysis for the official control of the compositional characteristics of honey").

Reducing sugars

The method is based on the reduction of Fehling's solution by the reducing sugars contained in a diluted solution of honey, in the presence of methylene blue as indicator [116].

Water content

The determination of the water content in honey is important to verify the conservation and to define its characteristics; its quantification is performed indirectly by measuring the refractive index of the honey or by thermogravimetric analysis.

Ashes and water-insoluble substances

Today these informations are obtained from thermogravimetric analysis.

Acidity

It is defined as "acidity of an aqueous solution of honey titratable with a solution of sodium hydroxide up to pH 8.5" and it's determined by a potentiometric titration.

Diastatic Index

Spectrophotometric determination of the amount of residual starch of a solution placed in incubation under standardized conditions with the solution of honey to be measured.

5-Hidroxymethylfurfural (HMF)

The official methods for *International Honey Council* (IHC) are the White method, the Winkler method and the chromatographic method (this latter was used):

CHROMATOGRAPHIC METHOD (HPLC)

This method involves the dissolution of 5g of honey in 50 mL of. After filtration, the solution is injected into a HPLC equipped with UV detector and an isocratic elution using as mobile phase a solution of 90% water and 10% methanol at a flow rate of 1 mL / min is carried out.

The hydroxymethylfurfural (HMF) content is then determined, using the internal standard method, by measuring the areas of the peaks of the analyte and the IS at 285 nm.

To obtain an accurate quantification, it is very important not to leave more than an hour and a half between sample preparation and its injection into the column, since problems can possibly arise from its degradation: to overcome this drawbacks, it is possible (according to the method of White) to add the Carrez solution.

The comparison of the three methods [116] pointed out that there is a discordance for low concentrations of HMF (for a HMF content of about 5mg/kg of the Winkler method gives higher concentration values than the other two), while for high concentrations (20 to 40 mg /kg) they are all concordant. From these considerations, it follows that any of the three methods can be used indiscriminately for the determination of the honey quality, due to their agreement around the legal limit (if we consider the parameters IHC, in fact, the upper limit is 80mg/kg for all types of honey); the use of the Winkler method is, however, recommended only in the cases where it is not possible to apply the others, because *p*-toluidine is carcinogenic.

5.3 DETERMINATION OF REDUCING SUGARS, WATER CONTENT AND 5-HMF

For the development of the method for the determination of reducing sugars and water content 14 samples of honey of different botanical origin were analyzed: three of acacia honeys, three wildflower honeys, one mountain wildflower honey, one orange honey, one linden honey, two chestnut honey, and two eucalyptus honeys.

On the other hand, for the determination of 5-HMF, 13 samples of uni-and multi-floral honey of different botanical origin were tested: four wildflower honey, two chestnut honey, two eucalyptus honey, one of acacia honey, one of linden honey, one of orange honey.

5.3.1 REDUCING SUGARS

A reducing sugar is a sugar which, put in solution, presents a ketone group or an aldehyde group in its open form. In solution, an equilibrium is established in which sugars are present both in their cyclic form and in their open-chain form. In particular, while the aldoses can be oxidized directly, the ketoses must first be transformed into aldehydes through a tautomerism that leads to formation of a carbonyl group at the end of the chain. As far as honey is concerned, the reducing sugars include glucose and fructose, while sucrose is not part of this category: from the standpoint of legislation, it is precisely this differentiation underlying the choice of this index as a quality criterion. In fact, a reducing sugar content below the legal limit is indicative of fraudulent addition of sugars, such as sucrose, to the product.

a) Reagents and preparation

Fehling's reagent

Fehling's reagent is a reagent used to identify organic compounds, especially sugars, with a reducing nature. This reagent can be used both in qualitative and quantitative analysis, as it is able to completely oxidize all reducing species.

The Fehling's reagent is formed by the union of two solutions called:

Fehling A: consisting of copper sulphate pentahydrate (69.278 g / L);

Fehling B: composed of potassium sodium tartrate (346 g / L) and NaOH (100 g / L).

The copper is maintained in solution due to the complexing effect of the tartrate, in the absence of which otherwise, it would precipitate as hydroxide, considered the basicity of the environment.

Solution of methylene blue

Methylene blue is a heterocyclic compound which at room temperature appears as a crystalline solid of dark green colour while dissolved in aqueous solution takes the dark blue colouring. This compound is widely used as a redox indicator, as its two forms, oxidized and reduced, have a different coloration: in particular, the oxidized form is intensely coloured in blue, while the reduced form is colourless. The potential of the transition depends on the pH and is equal to 0.53V at pH = 0, and decreases to 0.01V at pH = 7.

For the titration of sugars with Fehling method, the solution of methylene blue was prepared at a concentration of 1 g/L.

Preparation of standard solution of inverted sugar

9.5 g of pure sucrose were weighed and dissolved into 5 mL of concentrated hydrochloric acid; the resulting solution was then diluted with water to 100 mL. The acidified solution was then stored for several days at room temperature (about 3 days between 20 °C and 25 °C) and then diluted to 1000 mL. Immediately before being used, a volume of this solution was neutralized with a 1 M solution of sodium hydroxide and diluted to the required concentration (2 g/L) for the standardization.

Preparation of the test sample

About 2 g of homogeneous sample of honey, accurately weighed (W), were collected and dissolved in distilled water, the resulting solution was diluted to 100 mL in a volumetric flask. Successively, a further 1:4 dilution was performed, taking 50 mL of this solution and diluting them to 200 mL.

b) Standardization of Fehling's reagent

This step is the confirmation that the reagent is not altered and titrates exactly the amount of reducing sugars estimated. To do this, the title of the reagent Fehling solution A was checked, verifying that 5.0 mL mixed with 5.0 mL of solution B, react completely with 0.050 g of inverted sugar contained in 25 mL of diluted standard.

c) Preliminary titration

It is appropriate that the total volume of the solution at the equivalence point is 35 mL. For this purpose, it is normally necessary to add a certain volume of water before the start of the titration. To determine the volume of water to be added, a preliminary titration was necessary. In particular, 5 mL of Fehling's solution A, to which 5 mL of solution B were added, were placed in a flask together with 7 mL of water and a few grains of pumice stone or other boiling regulator; successively, 15 mL of the diluted solution of honey were added through the burette. The mixture was heated to boiling point on a plate, maintaining moderate boiling for 2 minutes. During boiling 3 drops of a solution of methylene blue were added and the titration was completed using small additions of solution of diluted honey until decolouration of the indicator in about 3 minutes. Defined as X mL the total volume of diluted solution of honey used for the preliminary titration, the volume of water to be added is equal to (25 - X) mL.

d) Determination of reducing sugars

Exactly 5 mL of Fehling's solution A were introduced, with a pipette, in a 250 mL flask, in which 5 mL of the solution B, (25 - X) mL of water, some grains of pumice and, with a burette, a volume of diluted honey corresponding to (X - 1.5) mL were added. The mixture

was then heated to boiling on plate, maintaining a moderate boiling for 2 minutes. During the boiling, 1 mL of the solution of methylene blue was added and the titration with the diluted solution of honey until decolouration of the indicator was completed in a total time not exceeding 3 minutes. Defined as Y mL the total volume of diluted honey used, the following formula was used for the calculation of the results, as shown in the Codex Alimentarius Standard for Honey, Ref No. CL 1993/14-SH:

$$C = \frac{4 * 500}{W * Y} \text{ (Eq. 5.3)}$$

Where:
C = g of inverted sugar per 100 g of honey, W = weight in g of honey sample, Y = volume in mL of the diluted solution of honey consumed in the determination.

5.3.2 WATER CONTENT THROUGH REFRACTOMETRIC ANALYSIS

By law, the determination of water content in honey is made using refractometric analysis. This technique is based on measurement of refractive index or the ratio between the speed of light in vacuum and that the substance to be analyzed, and can be used both in the case of solid and liquid or gas samples. The measurement was performed by an Abbe refractometer prism, which is a tool for liquid and solid samples. This type of refractometer is composed of two prisms, separated by about 0.15 mm. Within this thin layer, a small drop of the liquid to be examined is placed and the value of the refractive index is measured at the emission frequency of sodium. The value obtained must always be coupled to temperature [117].
According to the law, the refractive index of homogenized honey was determined at 20 ° C. If the determination had been made at different temperature, but near to 20 ° C, one would have to correct the value found using the following thermal coefficients: +0.00023 / ° C for temperatures above 20 ° C and -0.00023 / ° C for temperatures below 20 ° C. The percentage of water content was obtained from the following table of correlation.

Table 5.2: values are indicated in percentage of water present in honey in function of the refractive indices obtained by refractometer at a temperature of 20 ° C

Refractive index (20 °C)	**water %**	**Refractive index (20 °C)**	**water %**	**Refractive index (20 °C)**	**water %**
1,5044	13,0	1,4935	17,2	1,4830	21,4
1,5038	13,2	1,4930	17,4	1,4825	21,6
1,5033	13,4	1,4925	17,6	1,4820	21,8
1,5028	13,6	1,4920	17,8	1,4815	22,0
1,5023	13,8	1,4915	18,0	1,4810	22,2
1,5018	14,0	1,4910	18,2	1,4805	22,4
1,5012	14,2	1,4905	18,4	1,4800	22,6
1,5007	14,4	1,4900	18,6	1,4795	22,8
1,5002	14,6	1,4895	18,8	1,4790	23,0
1,4997	14,8	1,4890	19,0	1,4785	23,2
1,4992	15,0	1,4885	19,2	1,4780	23,4
1,4987	15,2	1,4880	19,4	1,4775	23,6
1,4982	15,4	1,4875	19,6	1,4770	23,8
1,4976	15,6	1,4870	19,8	1,4765	24,0
1,4971	15,8	1,4865	20,0	1,4760	24,2
1,4966	16,0	1,4860	20,2	1,4755	24,4
1,4961	16,2	1,4855	20,4	1,4750	24,6
1,4956	16,4	1,4850	20,6	1,4745	24,8
1,4951	16,6	1,4845	20,8	1,4740	25,0
1,4946	16,8	1,4840	21,0		
1,4940	17,0	1,4835	21,2		

5.3.3 WATER CONTENT THROUGH THERMOGRAVIMETRIC ANALYSIS

According to the law, the thermo-gravimetric analysis is the technique that must be used to determine the amount of ashes and water-insoluble substances present in honey. Based on this consideration, in the present research tests were performed to verify if it was also possible to determine the water content through this technique. For this purpose, the instrument used was the thermo-balance.

Balances available for this kind of analysis have an operating range between 1 and 20 mg. In the thermo balance the sample is placed in the oven, while the rest of the device is thermally insulated. In thermo-gravimetric analysis, the instrument records the loss in mass of the sample, subjected to a specific temperature ramp. The instrument is then able to identify all those thermal processes that result in a loss of mass: because the loss of water due to volatilization represents just one of these processes detectable, it was decided to study the possibility of using the thermal analysis for the quantification of water.

For this purpose, three measurements for each sample (5 mg) were made in an inert and oxidant environment. Also, two different temperature ramps were tested, in order to get as much information on the components sought:

a) heating at a constant rate of 5 °C/min [118], corresponding to the optimal conditions for the determination of the ash content and substances insoluble in water;

b) a programmed heating at not constant speed to be able to better appreciate the initial step of the thermogram (and, in particular, that corresponding to the loss of water).

The obtained thermograms were then exported to ASCII form using the TADS tool software.

5.3.4 HYDROXYMETHYLFURFURAL

In the absence of the standard samples, the quantification of HMF in the samples mentioned in the previous paragraph was performed through the use of the HPLC method, in order to obtain the reference values for the construction and validation of the NIR method. In particular, it was decided to perform the quantitative analysis through the use of the external standard. For the construction of the calibration curve of the HPLC method a standard of

HMF of purity over 99% (Sigma Aldrich, St. Louis, MO) was used. A stock solution was prepared at a concentration 1 mg/mL by dissolving the appropriate amount of standard in distilled water. The hydroxymethylfurfural solution of known concentration (in the range of 0.2-10 ng/µL) necessary for the quantitative analysis through the method of the external standard were prepared by dilution from the stock solution.

In detail, the quantification of hydroxymethylfurfural in honey samples through the HPLC method was performed using the following procedure. After homogenization of the sample, about 5 g (with an accuracy of 0.01g) of honey were weighed, and transferred into a 50 mL volumetric flask. Distilled water was added to the volume expected to completely dissolve the honey. The obtained solution was filtered on 0.45 µm filters and 20 µL of the filtrate were then injected into HPLC system. The chromatographic analysis was performed using a HPLC system interfaced with a photodiode array detector (ThermoFisher, Waltham, MA) and equipped with a C18 reverse phase column (length 15 cm and internal diameter 4.6 mm), packed with 5 µm particles. The elution was conducted using an isocratic mobile phase composed of 90% of ultra-pure water (MilliQ) and 10% methanol (HPLC grade, Carlo Erba, Milan, Italy); both of the phases were acidified with 0.1% formic acid (Sigma Aldrich, St. Louis, MO). For detection and quantification, the wavelength of 285 nm, the value at which it is possible to appreciate a detectable signal of the analyte even at very low concentrations and without interference, was chosen.

In a second phase of the study, to expand the range of concentration for the calibration and validation of the NIR method, since the real samples showed a small range of values, additional samples of honey were then prepared at known concentration of analyte, adding an appropriate amount of standard of HMF.

5.4 ACQUISITION OF NIR SPECTRA

The acquisition was made through the use of a NIR spectrophotometer. A drop of each sample was put between two laboratory glass slides. Before this step, each sample was mixed to obtain homogeneity. The spectra were acquired in transflectance mode [119]. Each honey was sampled and analyzed 4 times. All acquisitions were performed with 82 scans. Once

recorded, the data were saved in. csv and .spa using the software Omnic and exported to Matlab.

5.5 RESULTS - OFFICIAL METHODS

5.5.1 DETERMINATION OF REDUCING SUGARS

To get reliable values about the amount of reducing sugars present in honey samples, the official method was applied. For each of the 14 samples, 3 solutions were prepared, so as to verify reproducibility of the results. Subsequently, the average and the standard deviation of the values obtained on each sample were calculated.

Table 5.3: Concentration (%)of reducing sugars in the analyzed honey samples. Results of individual analyses, average and standard deviation.

sample	1st analysis	2nd analysis	3th analysis	average	RSD %
01 wildflower	64.6	64.5	64.6	64.6	0.1
02 acacia	60.0	60.2	60.0	60.1	0.2
03 acacia	59.2	59.4	59.2	59.3	0.2
04 linden	66.6	66.6	66.7	66.6	0,1
05 chestnut	65.6	65.9	65.8	65.8	0.1
06 wildflower	66.1	66.3	66.2	66.2	0.1
07 orange	68.2	68.5	68.5	68.4	0.2
08 acacia	60.1	60.1	60.0	60.1	0.1
09 chestnut	60.0	59.8	60.0	60.0	0.2
10 eucalyptus	70.7	70.7	70.6	70.7	0.1
11 wildflower	64.6	64.7	64.8	64.7	0.1
12 wildflower	62.2	62.3	62.1	62.2	0.2
13 eucalyptus	65.6	65.5	65.5	65.6	0.1
14 wildflower	66.1	65.9	66.1	66.0	0.1

5.5.2 APPARENT WATER CONTENT

The apparent water content was measured both with the refractometric and with the thermogravimetric analysis. However, in the case of the thermograms, difficulties in the choice of inflection points were experienced and, therefore, it was decided to approximate the values and the RSD% was higher than the experimental variability of gravimetric analysis. The refractometric analysis was also made just for four of the fourteen samples because of the unavailability of the Abbe refractometer for the analysis of all of honeys. However, this type of analysis has confirmed the results on the samples analyzed using both techniques.

The quantities of water obtained from thermogravimetric analysis are shown in the following table.

Table 5.4: Water content (%) obtained by the thermogravimetric analysis and its corresponding relative standard deviation

sample	% of water	RSD
01 wildflower	15.0	1.0
02 acacia	15.0	2.0
03 acacia	16.0	2.0
04 linden	16.5	2.0
05 chestnut	17.0	1.0
06 wildflower	17.0	1.0
07 orange	16.0	2.0
08 acacia	17.5	1.0
09 chestnut	16.0	1.0
10 eucalyptus	15.0	1.0
11 wildflower	14.5	2.0
12 wildflower	15.0	1.0
13 eucalyptus	15.5	2.0
14 wildflower	15.0	2.0

5.5.3 DETERMINATION OF 5-HMF

The results obtained are reported in Table 5.5 (for each sample, three replicate measurements were performed and the values shown are the average and the relative standard deviation, respectively).

Table 5.5: Results of the quantification of HMF obtained by chromatographic method

sample	HMF (mg/kg)	RSD%
01 wildflower	8.55	7.44
02 acacia	7.75	6.98
03 linden	<LOQ	
04 chestnut	10.40	6.70
05 wildflower	<LOQ	
06 orange	26.95	6.56
07 chestnut	11.28	7.03
08 eucalyptus	23.31	6.31
09 wildflower	22.44	7.23
10 wildflower	19.79	6.45
11 eucalyptus	14.04	7.09
12 wildflower	14.93	7.32
13 wildflower	12.99	7.16

The legal limit for the content of HMF in honeys for consumption is of 40mg/kg (except for some honeys such as citrus fruits for which the limit is lowered to 15mg/kg): examining the values in Table 5.5, it can be seen that all honeys tested are perfectly within the limits established by law, except for sample number 6 (orange honey). However, a careful analysis of the data in Table also shows how the range of concentrations of HMF in real samples was rather limited - between 7.75 and 26.95 mg/kg - and this is insufficient to be used for the development of the NIR method. In fact, in order to build an accurate model, the set of standards for calibration must adequately cover the experimental domain (and especially, in order to be able to detect frauds, a representative number of samples with values close to or above the limits set by law are also needed).

Therefore, before proceeding with the subsequent NIR analysis, new samples were prepared by adding increasing amounts of HMF standard. In this way, it was possible to extend the range of concentration of the samples up to 107.09mg/kg of HMF. The concentration of analyte in this new series of samples is shown in detail in Table 5.6.

Table 5.6: Samples and HMF added

sample	HMF (mg/kg)	sample	HMF (mg/kg)
14A1	20.44	21A1	29.60
14B1	30.70	21B1	39.56
14C1	39.01	21C1	49.84
14D1	51.09	21D1	60.02
14A2	61.18	21A2	71.72
14B2	71.89	21B2	80.30
14C2	79.08	21C2	92.33
14D2	88.36	21D2	101.75
22A1	26.58	19A1	33.35
22B1	33.70	19B1	43.04
22C1	44.71	19C1	54.67
22D1	54.72	19D1	64.64
22A2	63.94	19A2	74.07
22B2	75.43	19B2	80.75
22C2	85.15	19C2	92.13
22D2	95.27	19D2	104.55
23A1	24.69	16A1	37.00
23B1	34.65	16B1	46.49
23C1	46.11	16C1	56.47
23D1	53.78	16D1	67.28
23A2	47.40	16A2	76.13
23B2	76.35	16B2	88.01
23C2	82.63	16C2	96.45
23D2	96.20	16D2	107.09

The new set of samples obtained, allows both to have a greater number of honeys to be analyzed through the NIR spectroscopy to be used for the calibration and validation of the proposed method, and to cover a larger range of variability than it can be achieved with the starting samples of honey.

5.6 RESULTS – CHEMOMETRIC ANALYSIS

As already mentioned, one of the aims of this thesis was to study the possibility of developing alternative methods based on near-infrared spectroscopy for the determination of three important parameters of honey quality: water content, the amount of reducing sugars and the amount of idroxymethylfurfural.

For this purpose, the spectra of the samples were recorded in the range 4000-10000 cm^{-1}, according to the methods described in Section 5.4.

The "raw" spectral signals obtained are affected by various undesirable phenomena such as the presence of shift of the baseline, or multiplicative effects, probably due to scattering. These contributions to the signal constitute sources of undesirable variability (i.e., not bound to the analyte that is to be determined), so before chemometric analysis, a signal pre-treatment step was necessary.

The spectral data, after being exported, were converted into pseudo-absorbance units and pre-treated with the algorithm SNV (Standard Normal Variate) [115] to eliminate contributions to the variability of the signal, related to sources of spurious variability.

The set of NIR spectra measured, after SNV pretreatment, is shown in Figure 5.1.

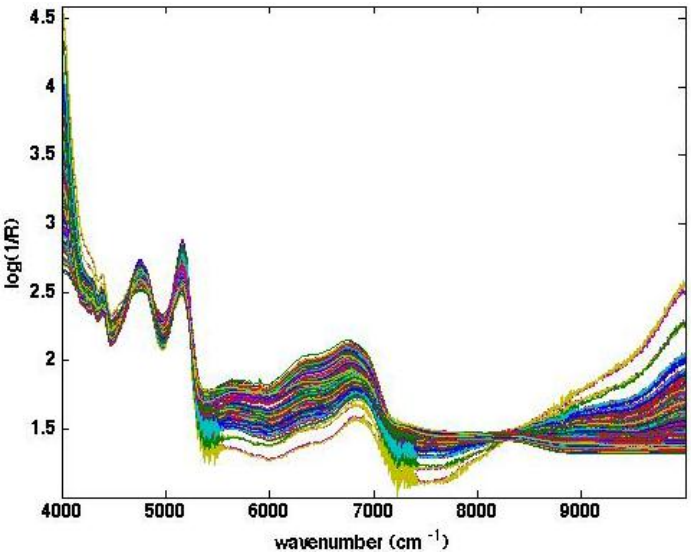

Figure 5.1: NIR spectra recorded, after pretreatment with SNV

The effects of the pretreatment can be visualized in a better way going to consider the graph of the first principal components obtained in the two cases. In fact, the principal component analysis (PCA) is a chemometric method for exploratory data analysis, which allows condensing the main sources of variability of the signal in a low-dimensional graphical representation (often two or three dimensions). The representation of samples in this reduced space (scores plot) provides useful information about the similarities and differences between samples. In particular, the next figures shown the scores plot obtained by projecting the samples on the space of the first two principal components calculated on the spectra without and with pretreatment, respectively.

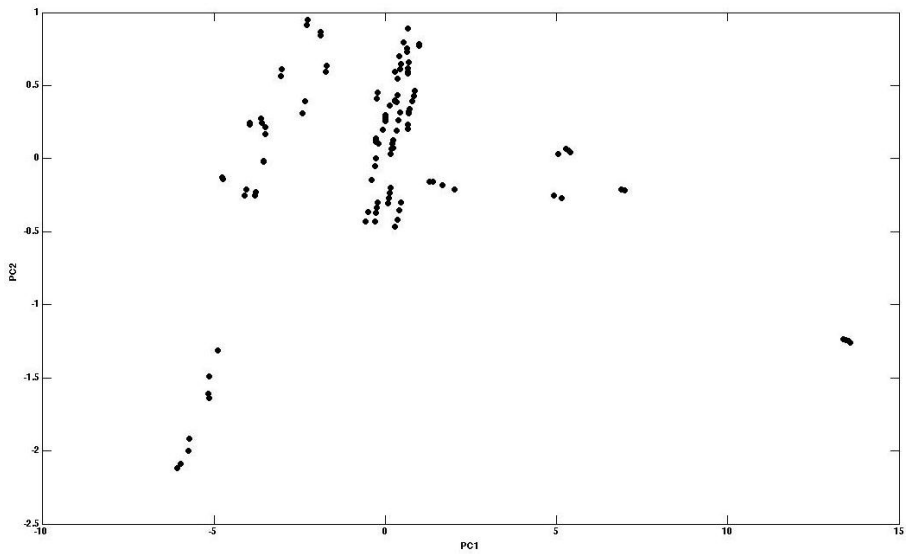

Figure 5.2: representation of the NIR spectra on the space of the first two principal components without pre-treatment.

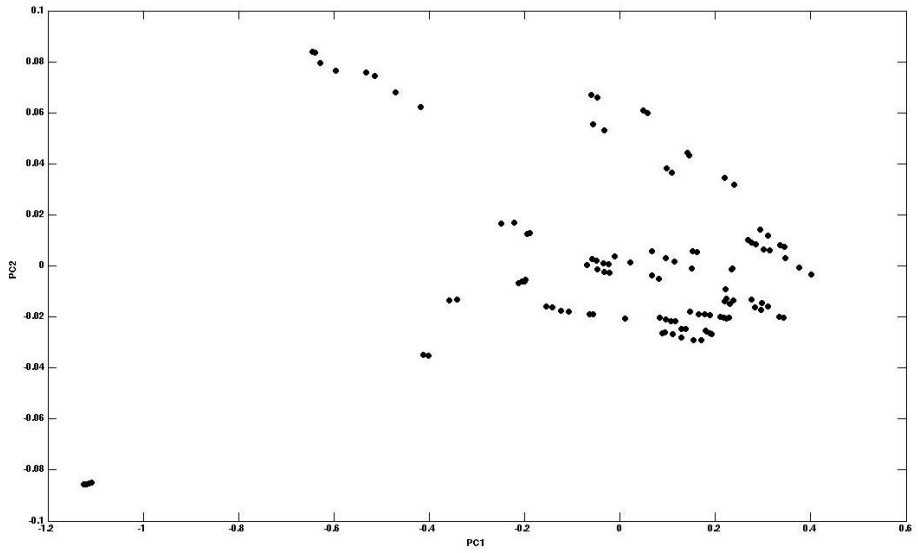

Figure 5.3: representation of the NIR spectra on the space of the first two principal components after pretreatment with SNV.

By the comparison between the two figures it can be observed how, without pretreatment, the differentiation between the groups of different measures result along the second principal component, while on the first principal component, which is the greatest source of variability of the signal, is linked to other (spurious variability). On the contrary, in figure 5.3 it is possible to observe how the measurements made on different honeys tend to position itself along the first principal component, indicating that the spectral pretreatment allowed to remove the undesirable sources of spurious variability.

The analysis of the graphs of the principal components in the case of pretreated signals data (Figure 5.3) also shows how some measures fall quite far away from most of the other, suggesting the possible presence of outliers in the spectral matrix.

For this reason, in the construction of the PLS calibration models for the prediction of the three indices of quality, a "robust" approach was used: "partial robust M regression (PRM)" algorithm was used to provide accurate results even in the presence of outliers [119].

The basic concept of the robust PLS calibration is that each of the data used for the construction of the mathematical model contributes to the definition of the model in a weighted way: in particular, the weights can take values ranging from 0 (element completely discarded) to 1 (data considered to be absolutely certain). In this context, it is necessary to specify why a measure may be abnormal in the case of multivariate calibration: outliers can

be characterized by rather high values of residues (for which the predicted y deviates much from the measured y), or they can be data for which the anomaly is linked to significant differences in the spectral signal (both in terms of form and intensity), such as the two points on the bottom left in the graph in figure 5.3. A further advantage of the robust approach to calibration is that, in addition to identifying the presence of outliers, is also able to provide information about type of anomaly that characterizes them.

On the basis of these considerations the construction of chemometric calibration models for the three parameters was performed. The set of spectral signals was divided into a training set and a test set, in order to have a set of measurements not used for the development of the calibration model. To do this, considering also the fact that the use of robust calibration methods require that all potential outliers are in the training set, the Kennard-Stone algorithm was used [120]. This algorithm operates the selection of the samples so that all the most diverse measurements (and thus, where present, also any anomalous data) are included in the training set.

5.6.1 DETERMINATION OF WATER CONTENT

First of all calibration model for the quantification of the water content in honey was developed. Results of thermogravimetric analysis were used as reference values for calibration, because it was not possible to use analyse by the refractometer all available samples. The choice of the optimum number of latent variables for the robust PLS model was performed using a cross-validation procedure and the resulting optimal complexity was 9 LV. The model developed resulted in a mean square error in calibration (RMSEC) of 0.165.

As shown, the important information that can be derived from the use of a robust method concerns the presence or absence of anomalous data (and thus the necessity or otherwise of the robust approach). This information can easily be represented a two-dimensional graph that relates the weights of the model. Indeed, it was previously described how the robust approach provides that to each sample a weight between 0 and 1 is associated, depending on its degree of anomaly: this weight is the resultant of two weights, related to the two different types of outliers described previously and indicated with the terms of "residual weight" (in the case of the residues) and "leverage weight" (in the case of the difference in the spectral domain).

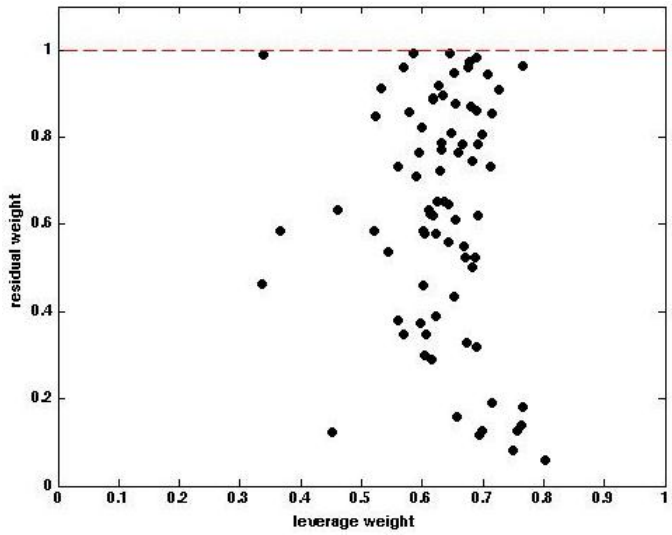

Figure 5.4: robust PLS model for predicting the water content - plot of the weights

As one can see in Figure 5.4, where the plot of the weights (residual and leverage) for the measures used for the development of the model is shown, different values fall relatively far from 1 in one direction or another (mainly due to the value of the residuals), thus confirming the validity of the initial assumptions concerning the possibility of anomalous data, and the need of a robust approach.

When the model was applied to the test set measures, a good mean square error in prediction (RMSEP), equal to 0.393, was obtained. The set of results in calibration and prediction is graphically shown in Figure 5.5, in which the measured and the predicted values of water content are compared, both for training and the test set.

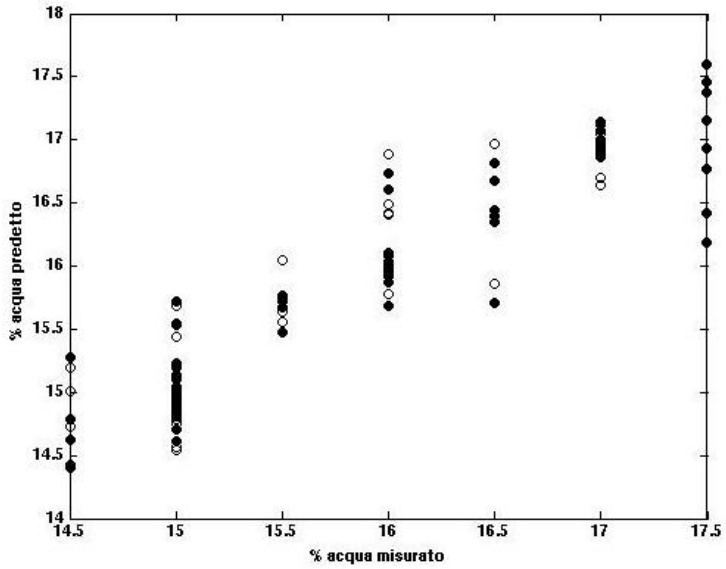

Figure 5.5: PLS calibration for predicting the water content in honey: observed vs. predicted plot for the training (full symbols) and the test set (empty symbols).

5.6.2 DETERMINATION OF THE CONTENT OF REDUCING SUGARS

A multivariate calibration model for the determination of the content of reducing sugars was performed. The same spectral matrix, pre-treated by SNV as described previously and divided into training and test set on the basis of the same criteria used for the quantification of water was used. For this purpose, the results of analysis performed with the Fehling's method were used as reference values.

Also in this case, for the construction of the calibration model a robust PLS approach was used, on the basis of the same considerations made for the determination of water content, since outliers could be present in the data matrix.

The best PLS model, chosen according to the results in cross-validation, consisted of 10 latent variables, and led to a mean square error in calibration (RMSEC) of 0.875.

The analysis of the weights performed as described previously, confirmed also for this second model the importance of having used a robust approach to multivariate calibration. Indeed, as shown in Figure 5.6, several weights are much smaller than 1, for both the leverage and the residue.

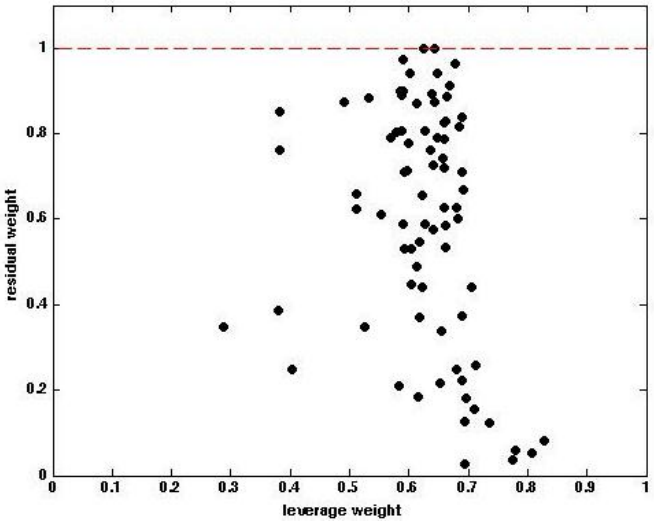

Figure 5.6: PLS model for quantification of the content of reducing sugars: plot of the weights of the model.

When the model was applied to the measures of the test set, it resulted in a mean square error of prediction (RSMEP) of 1.91. The comparison between the reference values and the values predicted by the model for the samples of the training and the test set is shown in Figure 5.7.

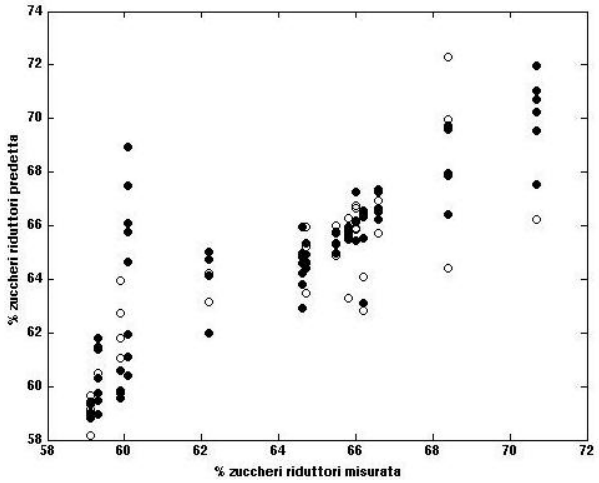

Figure 5.7: PLS calibration for the prediction of the quantity of reducing sugars in honey: observed vs. predicted plot for the training (full symbols) and the test set (empty symbols).

5.6.3 DETERMINATION OF 5-HMF

On the set of samples obtained by integrating the initial honey samples with further samples prepared with standard additions, the NIR analysis was carried out, in order to build a calibration model that could allow the quantification of HMF in a rapid, economic and non-destructive way.

As said, the set of samples was divided into two sets, the training set and the test set. In particular, to obtain a good representation of both sets, a splitting ratio of about 2:1 was chosen. Figure 5.8 shows the separation between training and test sets on the space of the first two principal components.

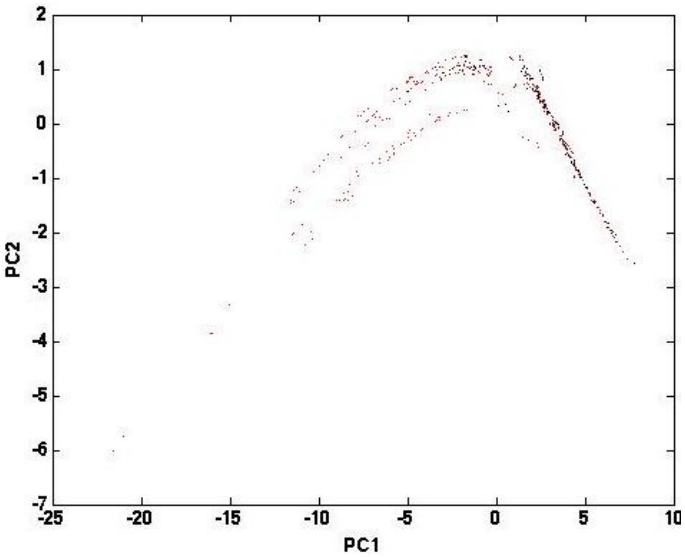

Figure 5.8: Distribution of training samples (red) and test (black) on the space of the first two PCs

The PRM algorithm was then applied to the NIR data, after transforming the variable Y (concentration of HMF) by calculating the square root: so, a model that would put in relation the measured spectral signal (after pretreatment SNV) with the quantity of analyte (5-HMF) was built. The model required the calculation of 11 latent variables (the choice of the optimal complexity was made on the basis of the error in cross-validation).

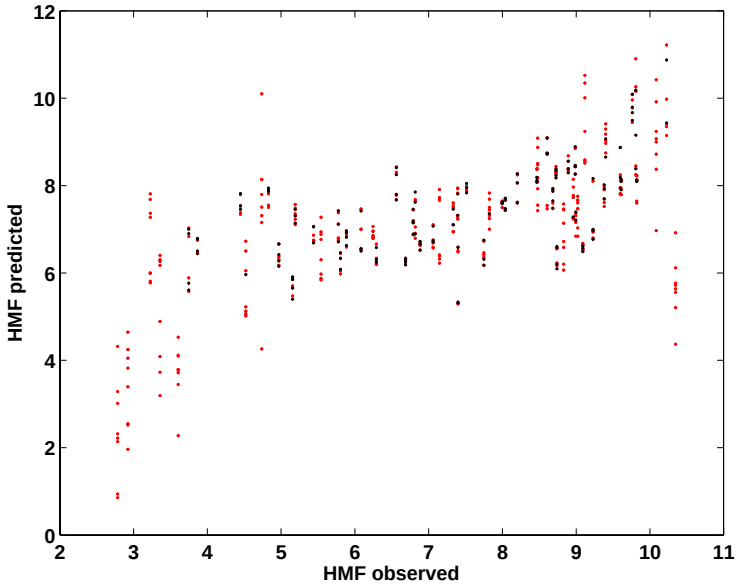

Figure 5.9: PRM model relating the spectral signal to the square root of HMF concentration: observed vs. predicted plot for the training (red) and the test set (black)

As it can be observed in figure 5.9, by using the PRM method relatively good predictions were obtained. The improvement with respect to the outcomes obtained by standard PLS due to the use of robust methods may indicate the presence of outliers in the training set. A confirmation of this hypothesis can be found by examining the plot of the weights.

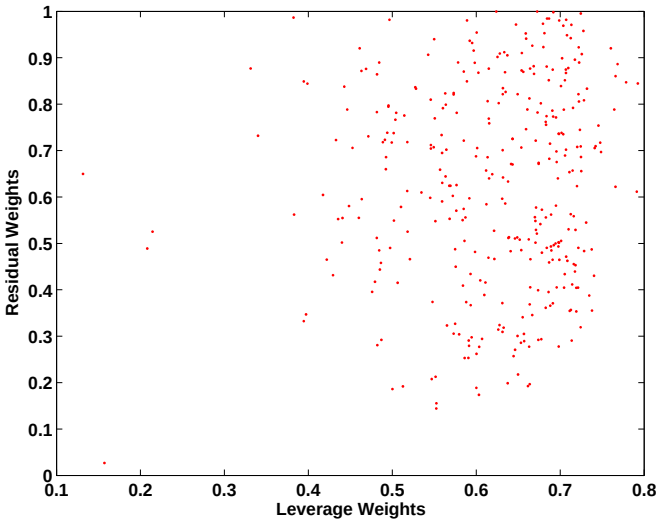

Figure 5.10 – PRM model after processing the square root of y: graph of the weights of the model.

As one can see from the graph, different samples of the training set are far from the vertex at the top right of the graph, corresponding to the optimal situation, reflecting the presence of some suspicious anomalous measures.

5.7 CONCLUSIONS

This research showed that FT-NIR spectroscopy coupled with chemometric analysis represents a valid alternative to the official methods of analysis for the determination of three indices of quality of honey, i.e., the content of water, of reducing sugars and of 5-idroxymthylfurfural. In particular, the results obtained through the spectroscopic method do not deviate too much from those obtained by the official methods, which have constituted the reference values for the setup of the calibrations. In this context, it was not possible to construct a regression model for the prediction of the ash content, although the data were available, since the values obtained with the TGA were not considered reliable enough due to the high standard deviation observed.

From the experimental point of view the spectroscopic method has proved to be advantageous compared to the titrimetric method and the thermo-gravimetric method, since the analysis time were much shorter. The method based on NIR spectroscopy is non-invasive, is more economic and, from an environmental point of view, zero impact (since it does not require the use of reagents), according to the guidelines of green chemistry. The time, cost and environmental impact of refractometer analysis, however, were found to be in line with those of spectroscopic analysis.

Comparing the thermogravimetric analysis with the refractometric one, the results obtained showed that the former can achieve performances comparable to those of the latter considering, on the one hand, that the analysis times are much longer and, on the other hand, that this technique is required to find the ash content present in honeys and therefore it is an analysis that still has to be performed.

The results, however, were obtained on a reduced number of samples, and this didn't allow to build a general model for the analysis of honeys. The work showed, however, the existence of the possibility to realize a model for this type of analysis having a sufficient number of samples available.

Moreover, the possibility of quantifying the hydroxymethylfurfural in honey samples through the coupling of NIR spectroscopy and multivariate calibration method was studied. In this context, best results were obtained by replacing the concentration of HMF with its square root, suggesting that there are sources of interference in the spectral signal that make the quantitative relationship not linear. However, this model was not accurate enough to be adopted. On the other hand, the use of robust methods of calibration, limiting the impact of any anomalous data on the results, allowed to significantly improve the predictive ability of the models developed, reducing, at the same time, the error in the prediction of validation samples.

In conclusion, these results, in general, suggest that it is possible to develop a method that allows the quantification of HMF in honey samples in a rapid, non-destructive and economic way by using NIR spectroscopy. However, the fact that the method proposed does not possess yet the accuracy and precision required for its use in official contexts, shows how this study represents only a preliminary investigation which necessarily should be examined further: processing more samples and, in parallel, checking chemometric models of increasing complexity.

CHAPTER 6

SAFFRON: MAE-HPLC-DAD FOR THE DETERMINATION OF QUALITY

6.1 QUALITY OF SAFFRON

Saffron is also called "red gold of the East" and it is the most expensive spice in the world [121, 122], being already known by ancient people who appreciated it for its characteristic aroma, flavor and taste [123]. Saffron is produced from the dried dark-red colored stigmas of the flowers of Crocus sativus *L.* grown in many countries of the Mediterranean basin and southern Asia [124]. The determination of the geographical origin plays an important role for this product, since some producing countries, such as Spain, have an export volume which appears to be too big if it is compared with their production. The original Spanish saffron is a product of high quality, but its costs have forced many farmers to move in countries such as Turkey where labor is cheaper [125]. The other producers of saffron are Arzebaijan, Iraq, Syria, Jordan, Egypt (the latter three are still following the traditional crop), Kashmir, China, Lebanon (one of the oldest manufacturers), Morocco, Italy, France, Greece, Switzerland.

As mentioned, saffron is known to be the most expensive spice in the world due to the limited cultivation and low harvesting yield. Its high price gives rise to several problems of sophistication [126].

In order to contrast sophistications and adulterations, the importance of quality control of saffron is clear.

The chemical composition of saffron is the most important indicator of its quality and of its commercial value in accordance with ISO 3632-1 and 3632-2 (2003). In fact, ISO 3632 norm was specifically designed to prevent and combat frauds, this precious spice could be subjected to. The growing number of frauds of this product, and economic speculation on market prices reflects an increasing interest from international research institutes.

The International Organization for Standardization (ISO) has defined the quality of saffron establishing three different categories (I, II and III) defined by specific parameters and ISO normative establishes also the procedures for their assessment.

Table 6.1: Specific parameters for the quality of saffron

Characteristics	Specs			Test methods
	Category			
	I	II	III	
Moisture and volatile substances (% max.) Saffron in filaments Ground saffron	12 10	12 10	12 10	ISO/TS 3632-2:2003, Clause 7
Total ash (% max.)	8	8	8	ISO 928:1997. Clause 8, and ISO/TS 3632-2:2003, Clause 12
Soluble ash in acid solution (%, max.)	1,0	1,0	1,5	ISO 930:1997. Clause 7, and ISO/TS 3632-2:2003, Clause 13
Soluble extract in cold water (max.)	65	65	65	ISO 941:1980, Clause 7
1% E 1cm 257nm, dried basis: min. (maximum absorbance of the picrocrocin)	70	55	40	ISO/TS 3632-2:2003, Clause 14
1% E 1cm 330nm, dried basis: min. max. (maximum absorbance of the safranal)	20 50	20 50	20 50	ISO/TS 3632-2:2003, Clause 14
1% Dyeing power, E 1cm 440nm, dried basis. (maximum absorbance of the crocins)	190	150	100	ISO/TS 3632-2:2003, Clause 14
Soluble artificial colorants in acid solution	No	No	No	ISO/TS 3632-2:2003, Clause 16 and/or Clasue17

These guidelines provide analytical methods, reference values and propose the classification of this spice on the basis of the content in specific active principles.

The general chemical composition of this spice is known. Saffron is made from the dried stigmas of Crocus sativus flowers, but, among the various substances which are present in the product, those which mostly attracted the interest of some researchers are bioactive compounds endowed with promising biological activities: crocin, crocetin, picrocrocin and the most important component of the volatile fraction safranal [127]. Their formation occurs during the flowering period and they are derived from a single precursor, zeaxanthin, after enzymatic cleavage.

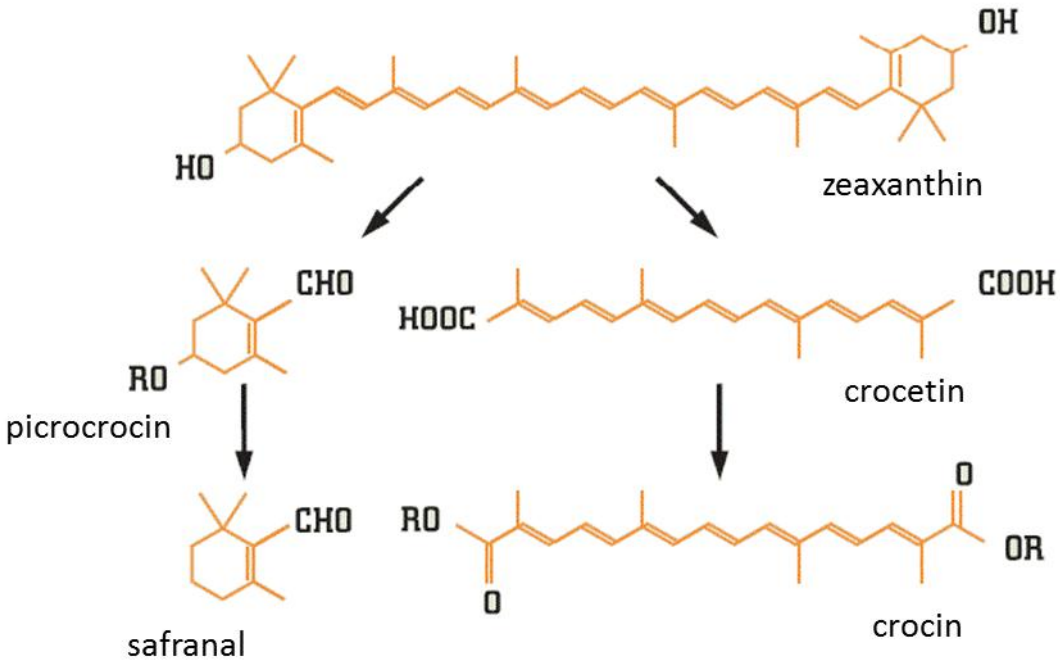

Figure 6.1: crocetin, picrocrocin, safranal and crocin biosynthesis

Safranal, the main responsible of the aroma of saffron spice, is easily obtained by hydrolysis of picrocrocin, its glycosidic derivative which in turn is the responsible of the taste of saffron.

The color of this spice is mainly due to glycosidic esters of crocetin, and in particular to crocin. All these components and their content determine the quality and the commercial category of legally traded saffron.

The quality of saffron depends on many factors, such as climate and soil of the area of production, and also by the mode of cultivation and drying.

Saffron production is constituted by four main phases. The first one is naturally the cultivation in specific conditions, the second and characteristic one is the manual harvest of the stigmas. The drying process represents the most critical phase of its production due to the registered differences among the producing countries. India, Iran and Morocco usually expose Saffron stigmas to direct sunlight, whereas Greece, Italy and Spain prefer drying processes indoor at higher temperature than ambient one. That's what has the strong impact on the amount of active principles and then on the quality of Saffron, due to the volatility and photodegradation processes. Lastly, the product could be marketed as dried filaments or ground as a powder.

Among the factors related to the quality of saffron, the determination of crocin, picrocrocin and safranal are the most important parameters, since these three compounds are analytes that most characterize the saffron. In the literature, different extraction techniques have been reported, on the basis of the component to be extracted.

The classical extraction method, described by ISO 2003, provides for the extraction of crocin, picrocrocin, safranal present in 0.5g of saffron using H_2O (1 L) and stirring the solution for 1 hour at room temperature. In the literature, there are also articles reporting ultrasonic extraction [128, 129].

As mentioned in the first chapter, a part of the thesis is focused on the development of an analytical method which could allow a fast, cheap and reliable analysis of the saffron permitting to control and to guarantee the quality of the product. Therefore a procedure for the determination of crocin, picrocrocin and safranal, based on the microwave assisted extraction (MAE) of the substances followed by HPLC analysis has been developed and optimized. In order to determine the quality of saffron, the method then has been applied for the analysis of samples coming from different manufacturers and from different geographical origins. Microwave-assisted extraction has never been applied to saffron for the recovery of its most important bioactive compounds and this technique provides in general a better isolation of the secondary metabolites after the complete swelling of the subcellular structures, faster times of extraction, reduced solvent waste and an accurate control of different parameter.

6.2 MICROWAVE-ASSISTED EXTRACTION OF CROCIN, PICROCROCIN AND SAFRANAL

For the microwave-assisted extraction of crocin, picrocrocin and safranal different solvents and mixtures of extractants were tested; instrumental parameters and time of extraction were also optimized.

6.2.1 SAMPLES AND CHEMICALS

Commercial standards of all trans-crocin (crocin-1, crocetin digentiobiose ester, 98%) and safranal (>88%) were purchased from Sigma-Aldrich (Italy), and picrocrocin (>98%) from Biotain PHARMA CO.LTD (China). Safranal was purified by column chromatography on silica gel (230-400 mesh, G60 Merck) using ethyl acetate:hexane (1:3) as the eluent. ^{1}H NMR, ^{13}C NMR and IR spectra of the purified product were in agreement with those reported in the literature. 4-Nitroaniline, used as internal standard (IS), was purchased from Sigma-Aldrich (Riedel-de Haën, Seelze, Germany). Methanol and Ethanol HPLC grade (<99.9%) were purchased from Carlo Erba. Ultrapure water generated by the MilliQ system (Millipore, Bedford, MA) was used.

One hundred six samples were collected from the best representative leading producers with particular interest towards Italian saffron from Sardinia and Latium. In particular, 20 samples from Greece, 25 samples from Turkey, 19 samples from Latium (Italy), 10 samples from Sardinia (Italy), 23 samples from Spain were collected. They were obtained under the guarantee of their quality in according to ISO 3632 guidelines. They were ground manually and sieved to obtain a uniform granulometry before performing the extraction/HPLC and the NIR spectroscopic analyses.

6.2.2 MICROWAVE-ASSISTED EXTRACTION PROCEDURE

Microwave-assisted extraction was performed by using a Biotage InitiatorTM 2.0 (Sweden). Ground saffron (approximately 10 mg) was placed in a sealed vessel suitable for an automatic single-mode microwave reactor (2.45 GHz high-frequency microwaves, power range 0-300 W) and a proper volume of solvent was added to the sample. The mixture was pre-stirred for 10 s and then heated by microwave irradiation for the time of extraction at 40 °C (irradiation power reaches its maximum at the beginning of reaction, then it decreases to lower and quite constant values). The internal vial temperature was controlled by an IR sensor probe.

Two of the parameters to be set to perform the microwave-assisted extraction are the temperature of the extraction solvent and the irradiation power. In order to avoid thermal degradation of the analytes, after preliminary tests conducted at different temperatures, it has been decided to set a constant temperature of 40 ° C for the entire duration of the extraction. this temperature was used for all extractions. In the next figures, as an example, are shown the graphs relating to temperature and irradiation power in function of extraction time.

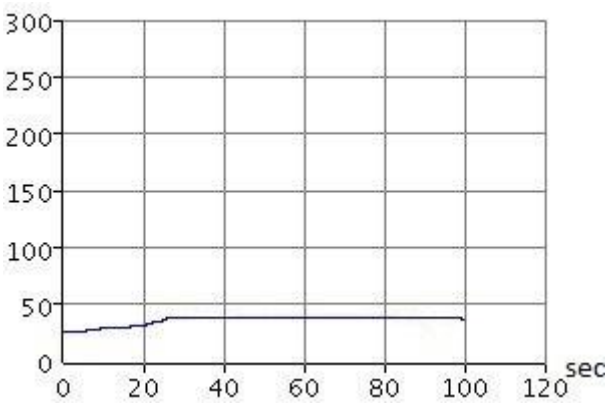

Figure 6.2: extraction temperature (° C) in function of time (s)

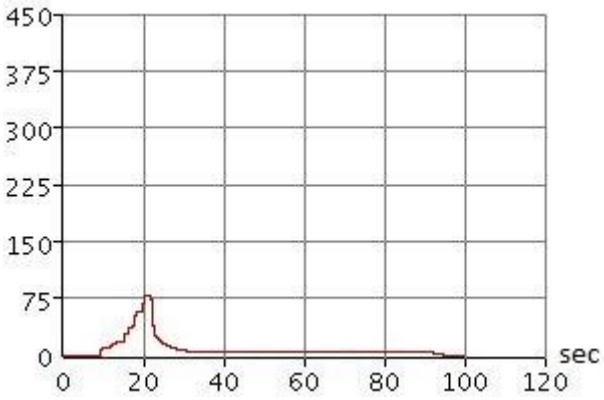

Figure 6.3: irradiation power (W) versus time (s)

In addition to setting the temperature and the irradiation power, the extraction time must also be set. In particular, 3 levels were considered for the extraction time: 1 minute, 10 minutes, and 19 minutes. Also, the following solvents were tested: MeOH, EtOH and mixtures H_2O:MeOH=50:50 and H_2O:EtOH=50:50. Lastly, for each solvent, the influence of the extraction volume was also tested by considering two different levels: 2mL and 10mL of solvent.

In order to optimize the extraction time, the type and volume of solvent, a full factorial design with 24 experiments (3*4*2=24) was carried out. The following table (6.2) schematically shows all the experimental variables and the levels considered for each of them.

Table 6.2: experimental variables and levels considerated

Solvents	**Extraction time**	**Solvent volume**
Ethanol (e)	1 minute (-1)	2 ml (-1)
Methanol (m)	10 minutes (0)	10 ml (1)
Methanol:H_2O (m/w)	19 minutes (1)	
Ethanol:H_2O (e/w)		

After the extraction of bioactive compounds, each extract was filtered through a PTFE filter (0.45 mm; Whatman-Merck, Darmstadt, Germany) and subsequently analyzed by HPLC-DAD.

6.2.3 HPLC-DAD ANALYSIS

Crocin, picrocrocin and safranal were determined by high performance liquid chromatography.

The extracts prepared according to what described in section 6.2.2 were analyzed by HPLC-DAD with a Thermo Quest Spectrasystem LC (Thermo Fisher Scientific, Waltham, MA) equipped with a P4000 pump, a UV6000 UV-Vis Diode Array Detector, and a SN4000 interface to be operated via a personal computer. Extracted compounds were separated using an Eclipse XDB-C18 analytical column (4.6x250mm, 5µm particle size; Agilent Technologies, Santa Clara, CA) protected by a guard cartridge of the same packing, operating at 25° C. Separation was carried out using gradient elution with a mixture of water (A):acetonitrile (B) (30–70% B in 20 min) at a flow rate of 0.9 mL min^{-1}. Injection volumes were 10 µL for all samples and standards. Multiwavelength detection was in the range of 200–550 nm and quantification was carried out by integration of the peak areas at 250 nm (picrocrocin), 310nm (safranal) and 440 nm (crocin).

6.2.4 OPTIMIZATION OF THE MICROWAVE-ASSISTED EXTRACTION

As anticipated in section 6.2.4, the operating conditions for the microwave-assisted extraction of the analytes from saffron samples were optimized using an experimental design. In particular, for each of the 24 designed experiments (for all extraction tests was used the same saffron sample), the extracts were subjected to HPLC analysis and the area of safranal, crocin and picrocrocin were integrated. Obviously, each area was normalized according to the area of the internal standard added (10µg) before each extraction. The conditions were optimized

to maximize both the quantity of each analyte extracted, and to maximize the concentration of each analyte extracted in the extracting solution. Indeed, on one hand it is important to assess the efficiency of extraction in order to develop an extraction method able to extract as much crocin, picrocrocin and safranal as possible. On the other hand, it is important to maximize the concentration of the analytes in the extract, to be able to analyze very dilute solutions and analytes in trace. Often, in fact even if the method developed allows to extract almost all of the analytes using big volume of solvents, it is necessary to add a step of concentration. In this specific case, however, it is not possible to make a concentration of the sample extract. In fact the use of a rotavapor or nitrogen flow to facilitate the elimination of the solvent leads to a significant loss of the more volatile compounds, such as safranal.

The next tables shown the normalized area (max=1) relative to safranal, crocin and picrocrocin. To determine which of the experiments was the best to extract the maximum quantity of safranal, crocin and picrocrocin, the area of each analyte was normalized in function of sample weight and area of the internal standard (10µg I.S. / 10mg of saffron). Then these areas (for each analyte) were normalized to 1.

Table 6.3: normalized quantity of extracted safranal; 1 means maximum quantity in the extract

		Extraction time		
	Area norm. safranal	1 minute	10 minutes	19 minutes
Extrac Solv.: EtOH	10ml EtOH	0,68	0,66	0,66
	2ml EtOH	0,61	0,63	0,61
Extrac Solv.: MeOH	10ml MeOH	0,73	0,68	0,68
	2ml MeOH	0,68	0,63	0,63
Extrac Solv.: MeOH:H_2O	10ml MeOH:H_2O	0,88	0,83	0,80
	2ml MeOH:H_2O	0,80	0,78	0,76
Extrac Solv.: EtOH:H_2O	10ml EtOH:H_2O	1,00	0,93	0,90
	2ml EtOH:H_2O	0,93	0,85	0,85

Table 6.4: normalized quantity of extracted crocin; 1 means maximum quantity in the extract

		Extraction time		
	Area norm. crocin	1 minute	10 minutes	19 minutes
Extrac Solv.: EtOH	10ml EtOH	0,13	0,13	0,14
	2ml EtOH	0,07	0,11	0,15
Extrac Solv.: MeOH	10ml MeOH	0,71	0,81	0,78
	2ml MeOH	0,61	0,71	0,68
Extrac Solv.: MeOH:H_2O	10ml MeOH:H_2O	0,93	1,00	0,86
	2ml MeOH:H_2O	0,86	0,92	0,80
Extrac Solv.: EtOH:H_2O	10ml EtOH:H_2O	0,76	0,82	0,78
	2ml EtOH:H_2O	0,73	0,82	0,78

Table 6.5: normalized quantity of extracted picrocrocin; 1 means maximum quantity in the extract

		Extraction time		
	Area norm. picrocrocin	1 minute	10 minutes	19 minutes
Extrac Solv.: EtOH	10ml EtOH	0,08	0,09	0,09
	2ml EtOH	0,04	0,07	0,10
Extrac Solv.: MeOH	10ml MeOH	0,75	0,70	0,69
	2ml MeOH	0,65	0,61	0,58
Extrac Solv.: MeOH:H_2O	10ml MeOH:H_2O	1,00	0,99	0,90
	2ml MeOH:H_2O	0,94	0,91	0,82
Extrac Solv.: EtOH:H_2O	10ml EtOH:H_2O	0,93	0,91	0,85
	2ml EtOH:H_2O	0,85	0,82	0,78

Table 6.3 shown that the best MAE to extract the greatest amount of safranal from sample is obtained extracting for 1 minute and using 10ml Et:H_2O=50:50. Table 6.4 shown that the best MAE to extract the greatest amount of crocin from sample is obtained extracting for 10

minutes and using 10ml MeOH:H_2O=50:50. Table 6.5 shown that the best MAE to extract the greatest amount of picrocrocin from sample is obtained extracting for 1 minute and using 10ml MeOH:H_2O=50:50.

Instead, to maximize the concentration we need to take account of the volume of solvent. The following tables refer to the maximization of concentration of the analytes in each extract. The area of each analyte is divided by the area of the internal standard (10µg I.S. / 10mg of saffron) and by the volume of extracting solvent used for extraction (areas are normalized to 1; max. area = 1).

Table 6.6: normalized concentration of extracted safranal; 1 means maximum concentration in the extract

		Extraction time		
	Area norm. safranal	1 minute	10 minutes	19 minutes
Extrac Solv.: EtOH	10ml EtOH	0,15	0,14	0,14
	2ml EtOH	0,66	0,68	0,66
Extrac Solv.: MeOH	10ml MeOH	0,16	0,15	0,15
	2ml MeOH	0,74	0,68	0,68
Extrac Solv.: MeOH:H_2O	10ml MeOH:H_2O	0,19	0,18	0,17
	2ml MeOH:H_2O	0,87	0,84	0,82
Extrac Solv.: EtOH:H_2O	10ml EtOH:H_2O	0,22	0,20	0,19
	2ml EtOH:H_2O	1,00	0,92	0,92

Table 6.7: normalized concentration of extracted crocin; 1 means maximum concentration in the extract

		Extraction time		
	Area norm. crocin	1 minute	10 minutes	19 minutes
Extrac Solv.: EtOH	10ml EtOH	0,03	0,03	0,03
	2ml EtOH	0,08	0,12	0,16
Extrac Solv.: MeOH	10ml MeOH	0,15	0,18	0,17
	2ml MeOH	0,67	0,77	0,74
Extrac Solv.: MeOH:H_2O	10ml MeOH:H_2O	0,20	0,22	0,19
	2ml MeOH:H_2O	0,93	1,00	0,88
Extrac Solv.: EtOH:H_2O	10ml EtOH:H_2O	0,17	0,18	0,17
	2ml EtOH:H_2O	0,80	0,89	0,84

Table 6.8: normalized concentration of extracted picrocrocin; 1 means maximum concentration in the extract

		Extraction time		
	Area norm. picrocrocin	1 minute	10 minutes	19 minutes
Extrac Solv.: EtOH	10ml EtOH	0,02	0,02	0,02
	2ml EtOH	0,05	0,08	0,11
Extrac Solv.: MeOH	10ml MeOH	0,16	0,15	0,15
	2ml MeOH	0,69	0,65	0,62
Extrac Solv.: MeOH:H_2O	10ml MeOH:H_2O	0,21	0,21	0,19
	2ml MeOH:H_2O	1,00	0,97	0,88
Extrac Solv.: EtOH:H_2O	10ml EtOH:H_2O	0,20	0,19	0,18
	2ml EtOH:H_2O	0,91	0,88	0,83

The MAE procedure which allows obtaining the highest concentration of safranal in the extract (table 6.6) corresponds to extracting for 1 minute and using 2mL of EtOH: H_2O. The MAE procedure which allows to obtain the highest concentration of crocin in the extract (table 6.7) corresponds to extracting for 10 minutes and using 2 mL MeOH:H_2O=50:50. The MAE procedure which allows obtaining the highest concentration of picrocrocin in the extract (table 6.8) corresponds to extracting for 1 minute and using 2 mL MeOH:H_2O=50:50.

As can be noted from previous tables we must use different mixtures of solvents (MeOH:H_2O for crocin and picrocrocin, EtOH:H_2O for safranal) and different extraction time (1 minute for safranal and picrocrocin, 10minutes for crocin) depending on which compound we want to optimize.

Regarding the volume of extracting solvent, the use of 2mL of solvent allows to obtain much higher concentrations of the analytes than using 10mL. Indeed the use of 10mL, even if the volume is 5 times greater than 2mL, does not lead to a comparable increase in the amount of analytes extracted.

On the basis of these results, for the analysis of safranal, crocin and picrocrocin in available saffron samples, it was decided to use the best conditions for the extraction of safranal (2mL EtOH:H_2O and 1 minute of extraction) because they provided a limited solvent waste balanced by a suitable extract concentration, reduced extraction times limiting the degradation of the active principles and the best recovery of safranal which is normally present in very low concentrations.

6.2.5 VALIDATION OF MAE-HPLC-DAD METHOD

The method developed, based on the microwave-assisted extraction was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), reproducibility and recovery (table 6.9).

Table 6.9: validation parameters – 2ml EtOH:H_2O and 1 min. extraction time; [a] Intermediate precision determined by different analysts on six separate weeks; [b] N=6

compounds	linearity (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	reproducibility (RSD%) [a]	recovery (%) [b]
safranal	0.5 – 55 R^2=0.9999	0.15	0.50	<3	87 ± 2
picrocrocin	0.5 – 50 R^2=0.9999	0.15	0.50	<3	80 ± 2
crocin	2.0 – 85 R^2=0.9999	0.15	0.50	<3	68 ± 2

As it is possible to see, crocin recovery due to the short extraction time is not so high; however, this analyte is still extracted in large amount thanks to its abundant presence in this spice.

The calibration curves for safranal, crocin and picrocrocin are reported in the next figures.

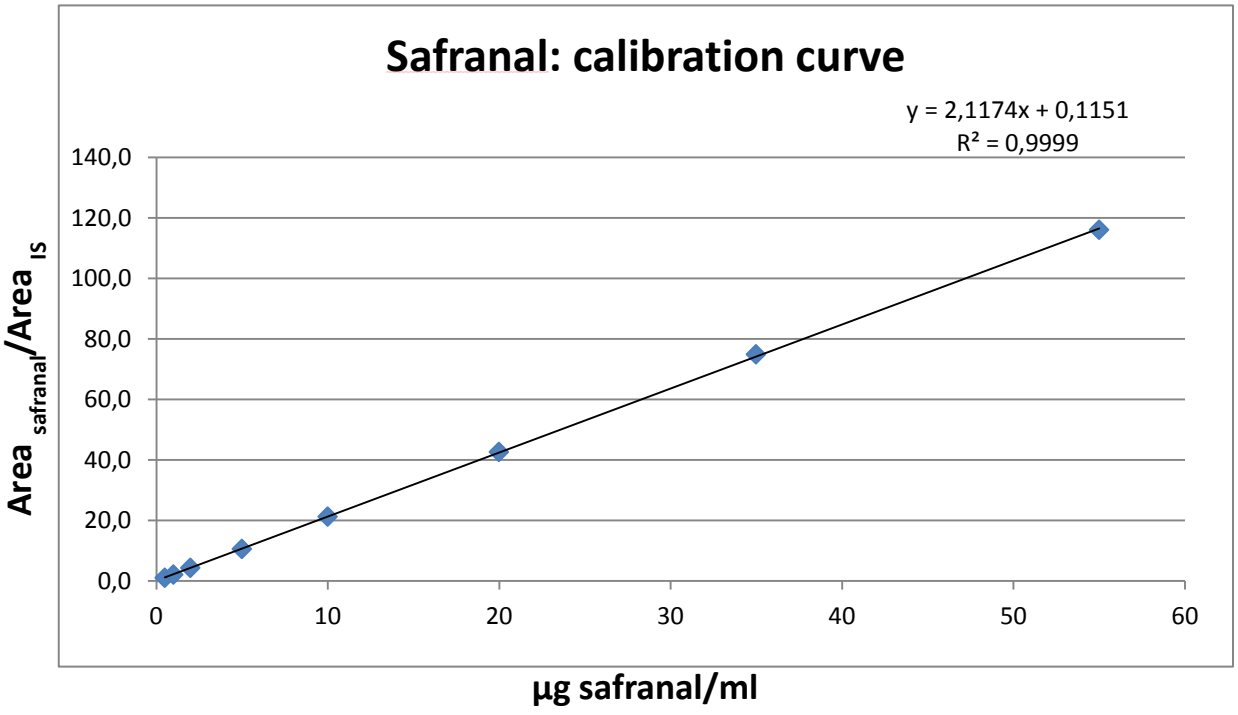

Figure 6.4: calibration curve of safranal

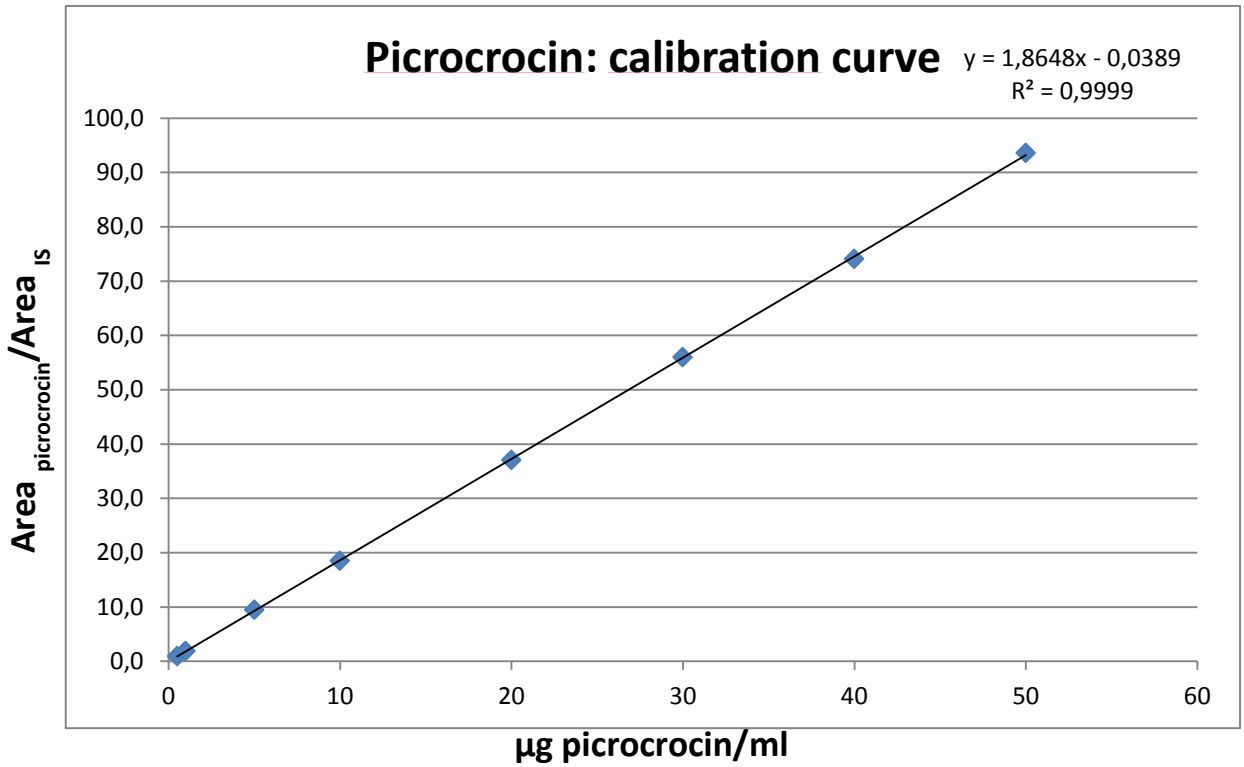

Figure 6.5: calibration curve of picrocrocin

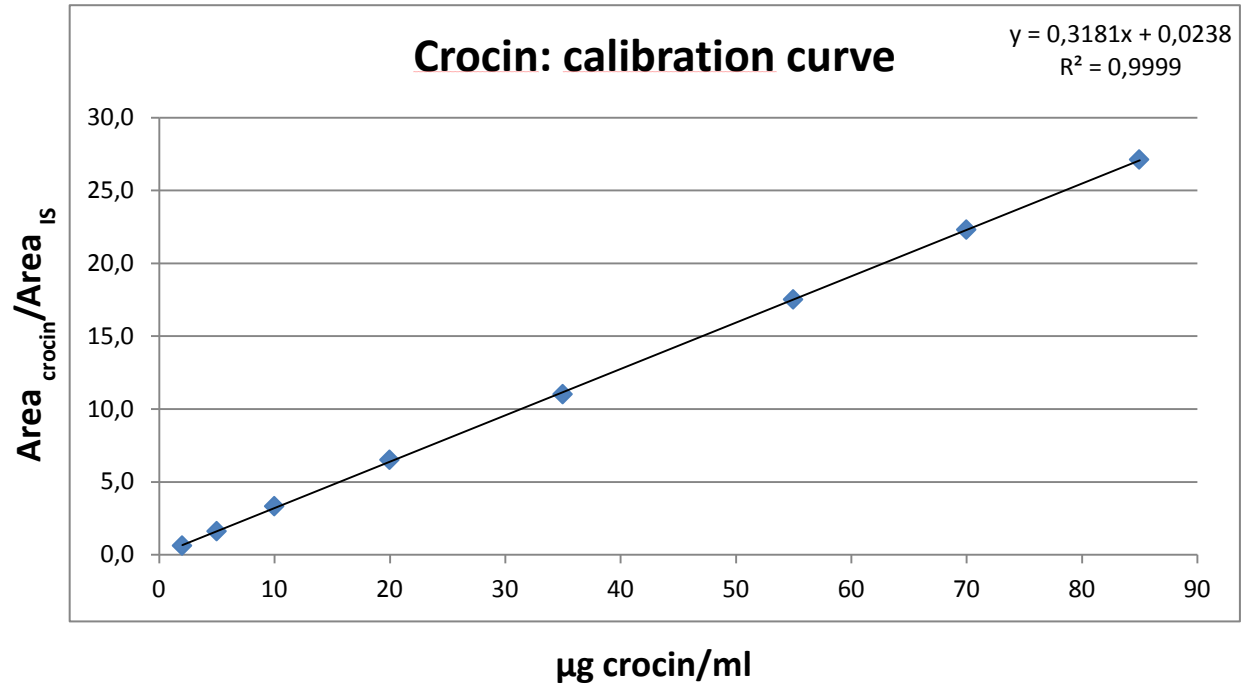

Figure 6.6: calibration curve of crocin

Since analyte-free saffron does not exist, LOD and LOQ were calculated as the average signal plus, respectively, 3 times and 10 times the standard deviation of a solution of EtOH: H_2O = 50:50.

LOD=0.15µg/ml; LOD=30µg/g

LOQ=0.50µg/ml ; LOQ=100µg/g

An RSD% <2 indicated that repeatability of procedure was satisfactory (N=6). Intermediate precision determined by different analysts on six separate weeks was also found satisfactory (RSD%<3).

Test for recoveries were performed on three different saffron samples (1 from Greece, 1 from Sardinia and 1 from Latium) comparing different extraction methods, such as ISO 2003 method [130]. Specifically the following extractions were compared for the recovery of safranal:

a) One cycle of extraction with 2ml (1x2ml) of EtOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
b) One cycle of extraction with 10ml (1x10ml) of EtOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
c) Two cycles of extraction with 10ml (2x10ml) of EtOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
d) Three cycles of extraction with 10ml (3x10ml) of EtOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
e) ISO 2003 Method: 0.5g saffron in one liter of H_2O, room temperature, with stirring for 1 hour.

Tests were carried out with more extraction cycles to assess the efficiency and relative recoveries for safranal. Furthermore the different methods were compared with ISO 2003 method [130].

Table 6.10: recovery of safranal

safranal																					
	samples	MW method (mg/g) [a]	MW method (µg/ml) [a]	MW method (mg/g) [b]	MW method (µg/ml) [b]	MW method (mg/g) [c]	MW method (µg/ml) [c]	MW method (mg/g) [d]	MW method (µg/ml) [d]	ISO 2003 (mg/g) [e]	ISO 2003 (µg/ml) [e]	recovery [a]	recovery [b]	recovery [c]	recovery [d]	recovery [e]
	GR01	4,31	21,55	4,71	4,71	4,96	2,48	4,96	1,65	4,22	2,11	0,87	0,95	1,00	1,00	0,85
	LA75	6,08	30,40	6,64	6,64	6,99	3,50	6,95	2,32	5,87	2,94	0,87	0,95	1,00	0,99	0,84
	SA33	3,21	16,05	3,51	3,51	3,65	1,83	3,70	1,23	3,10	1,55	0,87	0,96	0,99	1,00	0,84

[a] *1x2ml di EtOH:H_2O, 1min , 10mg di saffron , N=6 , RSD%<2*

[b] *1x10ml di EtOH:H_2O, 1min , 10mg di saffron , N=6 , RSD%<2*

[c] *2x10ml di EtOH:H_2O, 1min , 10mg di saffron , N=6 , RSD%<2*

[d] *3x10ml di EtOH:H_2O, 1min , 10mg di saffron , N=6 , RSD%<2*

[e] *ISO 2003 Method, N=6 , RSD%<3*

The following extractions were compared for the recovery of picrocrocin:

a) One cycle of extraction with 2ml (1x2ml) of MeOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
b) One cycle of extraction with 10ml (1x10ml) of MeOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
c) Two cycles of extraction with 10ml (2x10ml) of MeOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
d) Three cycles of extraction with 10ml (3x10ml) of MeOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.
e) ISO 2003 Method: 0.5g saffron in one liter of H_2O, room temperature, with stirring for 1 hour.
f) One cycle of extraction with 2ml (1x2ml) of EtOH:H_2O=50:50; 1 minute per cycle; 10mg saffron.

Tests were carried out with more extraction cycles to assess the efficiency and relative recoveries for picrocrocin. Furthermore the different methods were compared with ISO 2003 method [130].

Table 6.11: recovery of picrocrocin

picrocrocin																													
	MW method (mg/g) [a]	MW method (µg/ml) [a]	MW method (mg/g) [b]	MW method (µg/ml) [b]	MW method (mg/g) [c]	MW method (µg/ml) [c]	MW method (mg/g) [d]	MW method (µg/ml) [d]	ISO 2003 (mg/g) [e]	ISO 2003 (µg/ml) [e]	MW method (mg/g) [f]	MW method (µg/ml) [f]	recovery [a]	recovery [b]	recovery [c]	recovery [d]	recovery [e]	recovery [f]
samples																		
GR01	4,66	23,32	5,09	5,09	5,36	2,68	5,39	1,80	4,45	2,22	4,31	21,56	0,87	0,94	0,99	1,00	0,83	0,80
LA75	6,07	30,36	6,56	6,56	6,90	3,45	6,93	2,31	5,73	2,86	5,54	27,72	0,88	0,95	1,00	1,00	0,83	0,80
SA33	3,89	19,45	4,20	4,20	4,42	2,21	4,39	1,46	3,62	1,81	3,54	17,68	0,88	0,96	1,00	0,99	0,82	0,80

[a] *1x2ml di MeOH/water , 1min , 10mg di saffron , N=6 , RSD%<2*

[b] *1x10ml di MeOH/water , 1min , 10mg di saffron , N=6 , RSD%<2*

[c] *2x10ml di MeOH/water , 1min , 10mg di saffron , N=6 , RSD%<2*

[d] *3x10ml di MeOH/water , 1min , 10mg di saffron , N=6 , RSD%<2*

[e] *ISO 2003 Method, N=6 , RSD%<3*

[f] *1x2ml di EtOH:H_2O, 1min , 10mg di saffron , N=6 , RSD%<2*

The following extractions were compared for the recovery of crocin:

a) One cycle of extraction with 2ml (1x2ml) of MeOH:H_2O=50:50; 10 minutes per cycle; 10mg saffron.
b) One cycle of extraction with 10ml (1x10ml) of MeOH:H_2O=50:50; 10 minutes per cycle; 10mg saffron.
c) Two cycles of extraction with 10ml (2x10ml) of MeOH:H_2O=50:50; 10 minutes per cycle; 10mg saffron.
d) Three cycles of extraction with 10ml (3x10ml) of MeOH:H_2O=50:50; 10 minutes per cycle; 10mg saffron.
e) ISO 2003 Method: 0.5g saffron in one liter of H_2O, room temperature, with stirring for 1 hour.
f) One cycle of extraction with 2ml (1x2ml) of EtOH:H_2O=50:50; 10 minutes per cycle; 10mg saffron.

Tests were carried out with more extraction cycles to assess the efficiency and relative recoveries for crocin. Furthermore the different methods were compared with ISO 2003 method [130].

Table 6.11: recovery of crocin

crocin																									
samples	MW method (mg/g) [a]	MW method (µg/ml) [a]	MW method (mg/g) [b]	MW method (µg/ml) [b]	MW method (mg/g) [c]	MW method (µg/ml) [c]	MW method (mg/g) [d]	MW method (µg/ml) [d]	ISO 2003 (mg/g) [e]	ISO 2003 (µg/ml) [e]	MW method (mg/g) [f]	MW method (µg/ml) [f]	recovery [a]	recovery [b]	recovery [c]	recovery [d]	recovery [e]	recovery [f]						
GR01	12,93	64,63	14,25	14,25	15,00	7,50	15,03	5,01	12,47	6,24	10,22	51,10	0,86	0,95	1,00	1,00	0,83	0,68						
LA75	16,93	84,66	18,92	18,92	19,92	9,96	19,82	6,61	16,65	8,32	13,48	67,39	0,85	0,95	1,00	0,99	0,84	0,68						
SA33	11,54	57,71	12,55	12,55	13,35	6,68	13,42	4,47	11,27	5,64	9,13	45,63	0,86	0,94	0,99	1,00	0,84	0,68						

[a] *1x2ml di MeOH/water , 10min. , 10mg di saffron , N=6 , RSD%<2*

[b] *1x10ml di MeOH/water , 10min. , 10mg di saffron , N=6 , RSD%<2*

[c] *2x10ml di MeOH/water , 10min. , 10mg di saffron , N=6 , RSD%<2*

[d] *3x10ml di MeOH/water , 10min. , 10mg di saffron , N=6 , RSD%<2*

[e] *ISO 2003 Method, N=6 , RSD%<3*

[f] *1x2ml di EtOH:H_2O, 1min , 10mg di saffron , N=6 , RSD%<2*

6.3 DETERMINATION OF SAFRANAL, CROCIN, PICROCROCIN IN SAFFRON

The method (previously described and validated), which allows to obtain the maximum concentration of safranal in the extract, was then applied for the determination of constituents related to the quality of the saffron (safranal, picrocrocin and crocin) in all the available samples.

10 mg of ground saffron were placed in a 10 mL sealed vessel suitable for an automatic single-mode microwave reactor and 2mL of EtOH:H_2O were added to the sample. The extraction temperature was set at 40 ° C and the extraction time to 1 minute. The extract containing the analytes extracted was then filtered through a PTFE filter and 10µL were injected into the HPLC system. With the use of EtOH: H_2O and 1 minute of extraction, we obtained high recovery for safranal which is normally present in very low concentrations.

The following table shows the results obtained on 106 samples of saffron analyzed by MAE-HPLC-DAD.

Table 6.12: determination of crocin, picrocrocin and safranal in different saffron samples – GR means Greece, LA means Latium, SA means Sardinia, SP means Spain, TK means Turkey

sample	crocin (mg/g)	picrocrocin (mg/g)	safranal (mg/g)
GR01	15,03	5,39	4,96
GR02	14,03	5,11	5,45
GR03	14,48	5,31	4,81
GR04	12,60	4,45	4,30
GR05	13,02	4,83	4,37
GR06	13,47	4,88	4,35
GR07	12,31	4,35	4,38
GR08	12,96	4,66	4,27
GR09	11,08	3,75	3,72
GR10	13,45	4,60	4,73
GR11	15,32	5,54	5,21
GR12	12,44	4,65	3,92
GR13	17,04	5,99	6,48
GR14	14,26	5,16	3,89
GR15	14,56	5,18	4,81
GR16	14,10	4,91	3,90
GR17	15,41	5,45	4,81
GR18	13,83	4,74	4,49
GR19	14,04	4,87	4,26
GR20	15,26	5,41	4,15
LA75	19,02	6,93	6,99
LA76	16,24	6,05	6,10
LA77	17,82	6,41	5,55
LA78	18,17	7,97	5,71
LA79	17,41	7,29	6,24
LA80	14,24	5,12	4,58
LA81	16,23	5,86	5,76
LA82	7,98	2,75	2,73
LA83	19,69	7,94	4,33
LA84	14,22	5,02	3,06
LA85	15,28	5,56	3,24
LA86	14,04	5,19	3,29
LA87	17,90	6,61	2,56
LA88	13,54	4,77	2,97
LA89	17,08	7,32	2,73
LA90	15,13	5,45	2,57
LA91	15,72	13,00	3,26
LA92	14,66	5,12	2,80
LA93	14,62	5,21	3,20
SA33	13,42	4,42	3,70
SA34	13,31	4,62	3,84
SA35	14,25	4,27	2,53
SA36	14,42	4,45	4,44
SA37	5,15	1,46	2,75
SA38	14,92	4,61	3,77
SA39	5,08	1,39	3,54
SA94	3,62	0,83	2,36
SA95	3,79	0,80	1,15
SA96	3,59	0,84	2,24
SA97	3,55	0,81	2,52
SA98	16,97	4,74	2,11
SA99	18,41	5,33	2,36
SA100	18,77	5,03	2,29

sample	crocin (mg/g)	picrocrocin (mg/g)	safranal (mg/g)
SA101	18,93	5,32	2,38
SA102	16,91	4,52	2,07
SA103	15,27	4,22	2,06
SA104	15,08	4,05	1,80
SA105	16,56	4,71	2,19
SP21	15,05	4,59	2,99
SP22	15,03	4,66	2,96
SP23	10,00	3,12	0,94
SP24	11,57	3,65	1,02
SP25	16,19	5,20	2,09
SP26	16,17	5,21	2,18
SP27	17,51	5,61	1,91
SP28	16,80	5,48	1,91
SP29	17,23	5,81	2,02
SP30	18,33	6,69	1,86
SP31	17,80	5,59	2,37
SP32	17,16	5,75	2,46
SP40	5,37	1,37	1,86
SP41	12,81	4,10	1,85
SP42	15,80	5,17	2,43
SP43	14,87	4,80	2,22
SP44	13,40	4,26	1,40
SP45	14,36	4,90	1,59
SP46	14,20	4,28	1,56
SP47	14,73	4,49	2,06
SP48	13,05	4,45	1,78
SP49	13,90	5,25	1,93
SP106	7,16	2,08	1,55
TK50	3,98	0,62	0,30
TK51	3,94	0,59	0,24
TK52	3,99	0,66	0,33
TK53	3,80	0,58	0,24
TK54	3,61	0,52	0,18
TK55	3,68	0,57	0,24
TK56	4,71	0,82	0,36
TK57	3,52	0,53	0,19
TK58	3,60	0,54	0,20
TK59	3,43	0,54	0,19
TK60	3,62	0,52	0,18
TK61	4,69	0,64	0,32
TK62	3,49	0,55	0,20
TK63	3,60	0,56	0,22
TK64	3,61	0,55	0,21
TK65	3,78	0,56	0,22
TK66	3,57	0,53	0,19
TK67	3,68	0,58	0,24
TK68	3,40	0,55	0,20
TK69	3,78	0,55	0,23
TK70	3,45	0,51	0,17
TK71	3,62	0,54	0,21
TK72	3,50	0,55	0,21
TK73	3,70	0,55	0,21
TK74	3,51	0,54	0,21

The 106 saffron samples were analyzed and plotted in the space of experimental variables (safranal, crocin and picrocrocin). The next figure shows the distribution of the saffron samples analyzed.

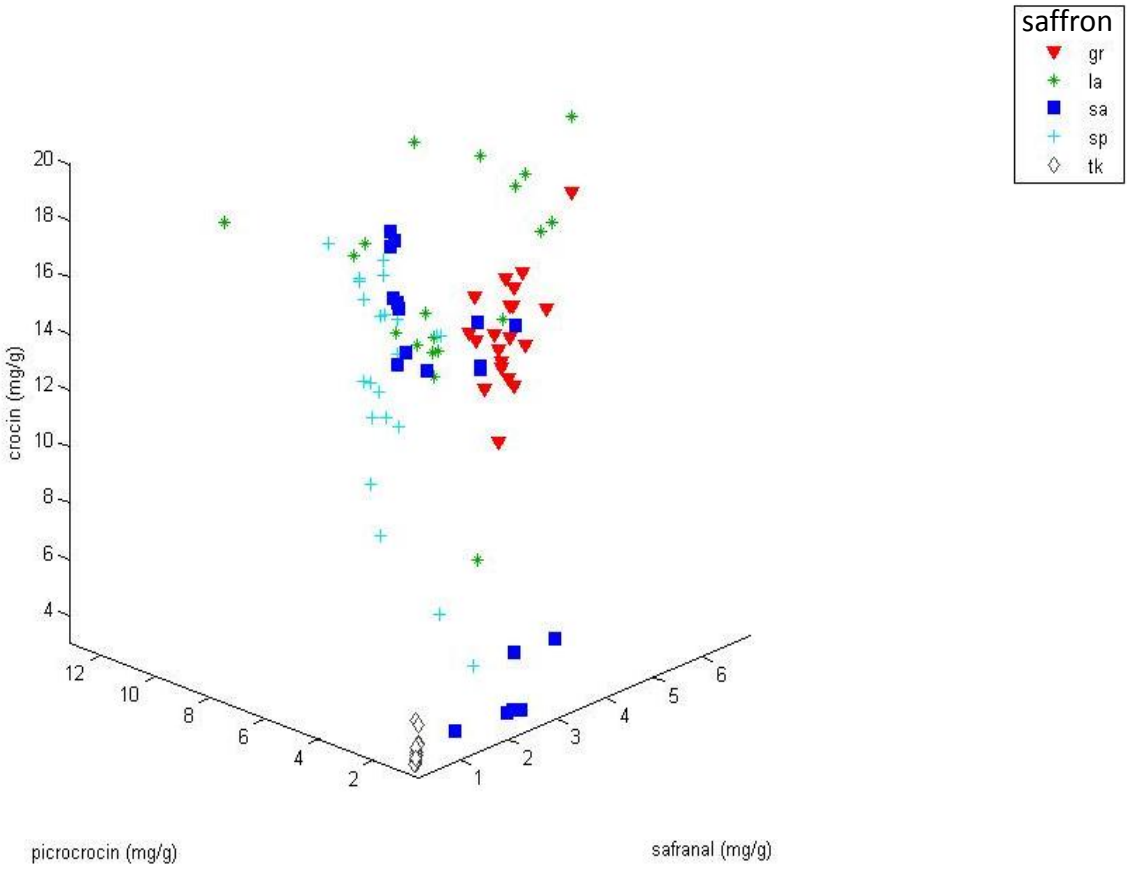

Figure 6.7: representation of the 106 samples in the space of the variables (safranal, crocin and picrocrocin)

Figure 6.7 shows how the Turkish samples are grouped in a clearly limited area and that they are relatively far from all the other samples produced in other geographical areas.

The graphical representation of the results also shows how the Greeks samples and the Spanish samples are well separated in the space of the three experimental variables.

The MAE-HPLC-DAD results collected on the available samples were also used to build a model to discriminate the 5 classes of samples (Greece, Latium, Sardinia, Spain, Turkey). The method used for discriminant classification was linear discriminant analysis (LDA) [131]. Aim of Linear Discriminant Analysis is to find one or more linear combinations of parameters which allow to discriminate optimally the various groups of samples. In this way it is possible to assign an observation (sample) in a given class on the basis of measurements of crocin, picrocrocin and safranal. In next table we report the error in cross-validation evaluated by linear discriminant analysis for each distinct saffron.

Table 6.13: LDA of saffron samples; the quantity (mg/g) of safranal, crocin and picrocrocin were used; cross validation (CV)= venetian blinds w/ 5 splits

	Greece	**Latium**	**Sardinia**	**Spagna**	**Turkey**
Error in Cross-Validation	1	13	13	4	0
number of samples	20	19	19	23	25

LDA provided a classification on the basis of the corresponding content of crocin, picrocrocin and safranal in different saffron samples. Saffron from Latium and Greece presented the bigger concentrations of crocin, picrocrocin and safranal. Saffron from Sardinia were split into two clusters with completely different amounts of their active principles. Spanish saffron is the most scattered and in general is characterized by a low concentration of safranal. Turkish saffron displayed the lowest concentrations of the active principles and this could be due to the specific drying procedure.

6.4 GEOGRAPHICAL ORIGIN BY NIR

The method developed, based on microwave-assisted extraction, allows a limited use of solvents and efficient use of saffron, allowing a rapid quality control. In fact, the amount of picrocrocin, crocin and safranal indicate the quality of saffron being related on taste, smell and color. The amount of crocin, picrocrocin and safranal are also related to the geographical origins of production. In fact, type of soil, climatic conditions, modes of production and storage, change in function of different geographical areas of production. Saffron is dried differently (shade, heating system, electric ovens, sunlight, etc.) in various regions of the world, and drying practices are known to affect the final composition of saffron. Crocins and picrocrocin compounds degrade naturally in the cells of stigmas during drying and storage [132].

Even if the MAE-HPLC-DAD method compared to other methods presents in the literature allows a rapid analysis and a limited use of solvents, a method based on NIR spectroscopy for discriminant classification of saffron was also developed. NIR spectroscopy is a non-destructive, non-invasive, rapid, and it does not require any pre-treatment of the sample. The are many advantages of its use, in addition to those already mentioned. From an environmental point of view, it results zero impact: saffron can be analyzed without any pretreatment and therefore without the use of solvents. NIR spectroscopic analysis is also economic because it does not need reagents.

6.4.1 ACQUISITION OF NIR SPECTRA OF SAFFRON SAMPLES

For the acquisition of spectra in the near infrared range, a Nicolet 6700 FT-NIR instrument (Thermo Scientific Inc., Madison, WI), equipped with a tungsten–halogen source and an InGaAs detector, was used. The spectra were acquired at room temperature and without any further sample treatment, in reflectance mode, through the use of an integrating sphere (Thermo Scientific Inc., Madison, WI). Operationally, for the acquisition of each spectrum, the proper amount of saffron (approximately 10 mg) was placed inside a cylindrical glass sample holder (19 mm internal diameter, 2.7 cm in height), which was then positioned on the hole of the integrating sphere. The spectra were acquired between 10,000 and 4000 cm^{-1},

collecting 82 scans at a nominal resolution of 4 cm^{-1}. For each sample four spectra were acquired for a total of 424 spectra (106*4). The data were then exported from Omnic Suite software (Thermo Fisher Scientific Inc., Waltham, MA) as an ASCII file, which was then imported into MATLAB (release R2009b, The MathWorks Inc., Natick, MA), for the successive chemometric analysis.

6.4.2 PLS-DA MODEL

The "raw" spectral signals obtained are affected by various undesirable phenomena, such as the presence of shift in the base line, or effects due to the scattering. Since these contributions to the signal are unwanted sources of variability (i.e. they are not related to the phenomenon of interest), before chemometric analysis, a pre-treatment of the signals was necessary. Therefore, after being exported, the spectral data were converted in pseudo-absorbance and pretreated with the SNV (Standard Normal Variate) algorithm [115]. After pretreatment, spectral data matrices were built by averaging the pretreated signals of the four replicated measurements for each sample. Figure 6.8 shows the set of NIR spectra measured after SNV pretreatment.

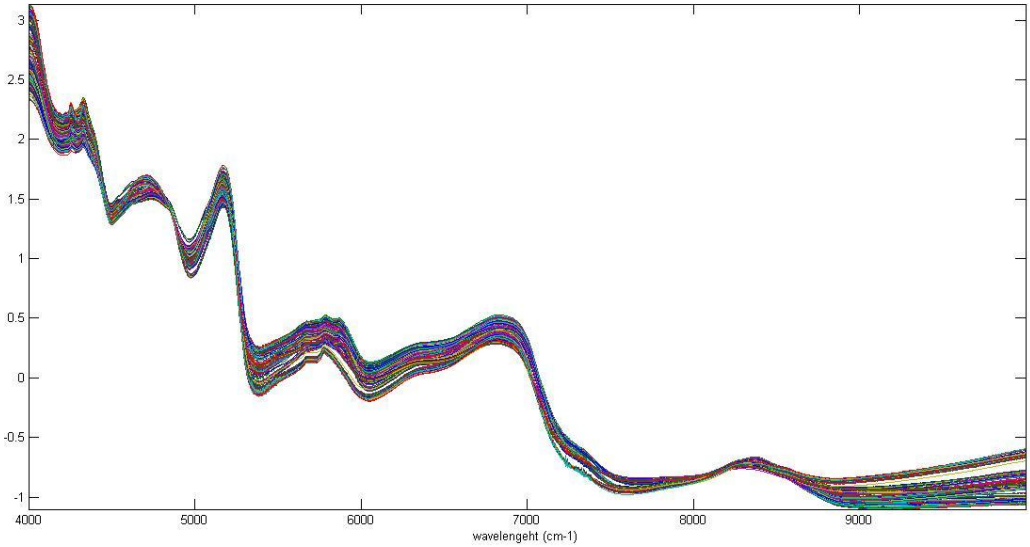

Figure 6.8: near infrared spectra collected on the saffron samples; after SNV pretreatment

The 106 samples were then plotted in the space of the principal components (PC). Next figure shown the samples produced in different ways and in different geographical areas, in the space of the first two PCs.

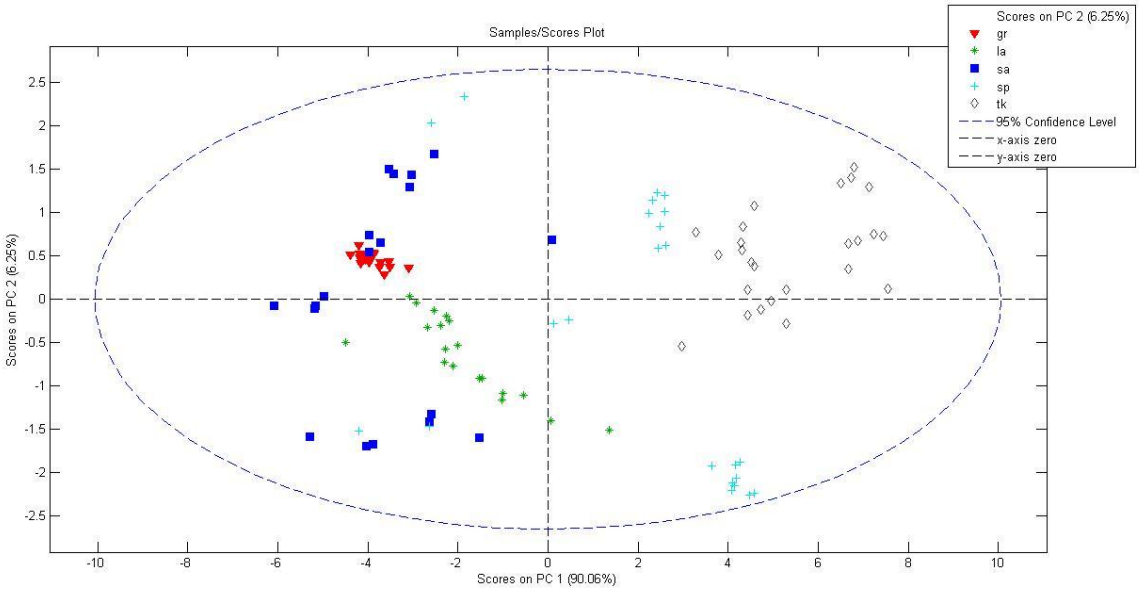

Figure 6.9: representation of the samples in the space of the first two principal components

The analysis of the principal components shows how the samples from the same geographical area of production are well grouped.

As can be seen from the representation of the 106 samples in the space of the safranal, crocin and picrocrocin variables (figure 6.7), also in this case (figure 6.9), with the NIRS data is possible to note for the Greek samples a cluster in a narrow space of the PC. Even the Turkish samples in the space of PC can be grouped together and separated from the other samples. The same analysis can be made for Latium samples, which form a distinct cluster from the other samples. With the NIR data, in addition to exploratory analysis, in order to discriminate the different geographical origins and production of saffron we developed a classification model (PLS-DA).

Table 6.14: PLS-DA model, latent variable=9, cross validation (CV)= venetian blinds w/ 10 splits

NIR data	Greece	Latium	Sardinia	Spain	Turkey
sensitivity cal.	1.000	1.000	1.000	0.913	1.000
specificity cal.	1.000	1.000	0.966	1.000	1.000
sensitivity CV	1.000	1.000	0.947	0.913	1.000
specificity CV	0.988	1.000	0.954	0.976	0.988

The low number of samples did not allow an external validation of the model. However, the model developed allows an almost perfect classification in cross validation of all the five available classes of samples.

6.5 CONCLUSION

A microwave-assisted extraction system for biologically active compounds has many advantages over other conventional extraction methods. Microwave-assisted extraction methods required shorter time, less solvents, provide higher extraction rates and better products with lower costs.

It can be concluded that microwave-assisted extractions provide significant advantages in terms of extraction efficiency and time savings.

The method developed allows high recovery, it is very reproducible and allows to analyze samples with very low concentration of safranal, crocin and picrocrocin. This method, thanks to the high concentration factor (only 2 mL of solvent are used), can be used for the analysis of compounds and derivatives with similar characteristics to safranal, crocin and picrocrocin even if they are present in very low concentrations. It may be used also for the analysis of other spices in which the amount of safranal, crocin and picrocrocin are very low. We obtained, with the use of microwave-assisted extraction, concentrations of extracts approximately 10 times higher compared to the ISO 2003 method and to methods which use ultrasounds for the extraction [129,133,134]. Moreover, with the method developed, it is possible to simultaneously analyze both safranal, and crocin/picrocrocin, while the works reported in the literature are focused on the determination of either safranal or crocin and picrocrocin [123,128].

Although a perfect geographical classification could not be performed only with the quantitative analysis of crocin, picrocrocin and safranal, we can assert by graphical analysis of the samples in the space of these three parameters (figure 6.7) and by the results of the linear discriminant analysis in cross validation (table 6.12), that the content of these bioactive compounds is strongly linked to the production area.

A discriminant method of classification faster than HPLC-DAD analysis was developed. The analysis of the NIR spectra of the saffron samples allowed to discriminate in a rapid, non-destructive and zero impact way, samples from the different production areas such as Turkey, Greece, Spain and Italy (Latium and Sardinia).

In the future, the method based on NIR spectroscopy will be validated with samples which will be harvested in 2014: doing so it will be possible to validate the PLS-DA model developed with a test set does not used to build the model.

CHAPTER 7

WATER: DETERMINATION OF BENZOTRIAZOLES IN WATER SAMPLES

7.1 INTRODUCTION

Benzotriazole derivatives are categorized as high production volume chemicals, being complexing agents widely used as anticorrosives (e. g. in engine coolants, aircraft deicers and antifreeze liquids) and for silver protection in dish washing liquids [135,136]. Toxicological studies have demonstrated that they might be hazardous to plants [137,138], mutagenic in bacteria cell systems [138] and toxic to some microorganisms [139]. Moreover, 1H-benzotriazole (BTri) has been classified as a suspected human carcinogen by the Dutch Expert Committee on Occupational Standards [138]. In the environment, benzotriazoles are considered as emerging pollutants [135,136], with sewage treatment plants (STPs) representing one of the most important discharge sources of these compounds into the aquatic media [140-142].Thus, they have been detected in different aquatic compartments, such as surface, ground or wastewater [135,136], sludge [143,144] and sediments [144]. Also, benzotriazoles appear in indoor environments (indoor dust) [145], and even in human urine [146].

Due to their polar character (log K_{ow} values from 1.44 to 2.25), high water solubility and low volatility, liquid chromatography (LC), usually coupled to mass spectrometry (MS), has been

the preferred technique for their sensitive determination in environmental samples during last years [136]. Most water samples analysis have been carried out using triple quadrupole LC-MS/MS instruments, achieving methodological LOQs in the low ng L^{-1} [140,147-150]; furthermore, other types of mass analyzers, such as LTQ FT Orbitrap MS [151], HRMS [152] and QTOF MS [153], have also demonstrated their suitability for benzotriazole determination in combination with LC as separation technique. Limited performance of gas chromatography (GC) methods for benzotriazole compounds has been overcome by the use of ionic liquid stationary phases coated columns [154], derivatization processes, such as methylation [155,156] or acetylation [157], and the use of two-dimensional gas chromatography [156,158]. But for now, no simple analytical methodologies, based on the use of a routine laboratory affordable GC-single quadrupole MS instrument, have been developed, able to reach LOQs comparable to those provided by LC-MS/MS methods and an improved isomer resolution.

Regarding sample preparation, solid-phase extraction (SPE), using conventional hydrophilic-lipophilic balanced polymeric materials such as OASIS HLB [140,146-148,151] or Strata X [154,156], remains as the most popular concentration technique for benzotriazoles determination in water samples. SPE, based on reversed-phase polymers, is also the preferred approach to carry out multiresidue water sampling campaigns in which these emerging pollutants are often included [159-162].

Despite microextraction techniques potential advantages, such as miniaturization, low solvent consumption and high selectivity [163, 164], they have just been scarcely investigated for the extraction and preconcentration of benzotriazoles. As regards solid-phase methodologies, stir-bar sorptive extraction (SBSE) has been tested for the determination of BTri in ultrapure water using coating materials with different polarities, such as polydimethylsiloxane (PDMS), polyacrylate (PA) with a proportion of poly(ethylene) glycol (PEG) and a PEG modified silicone. In all cases, the extraction efficiency for BTri remained below 1%, for 50 mL of ultrapure water, after sampling during 4 hours [165]. Benzotriazoles have also been successfully concentrated from water samples using a disposable polar membrane of polyethersulfone [153]. However, the sample preparation method required 6 hours to achieve equilibrium conditions. Slow extraction kinetics, which are characteristic of solid-phase microextraction techniques, can be overcome by some liquid-liquid microextraction methodologies, such as dispersive liquid-liquid microextraction (DLLME) [163]. Following the first report by Assadi and coworkers [166] in 2006, a high number of DLLME applications have been published. Some of them, as well as the most outstanding trends in

DLLME, have been revised in a recent review [167]. To the best of our knowledge, the only application of DLLME to benzotriazoles analysis considered tri-n-butylphosphate as extractant, with concentrated species determined by LC with fluorescence detection and LC-MS/MS [168]. Obviously, the above extractant was unsuitable to be used in combination with GC-MS determination.

Therefore, main aims of this work are (1) the development of a simple, easy, highly efficient, environmental friendly and low cost sample preparation proposal, based on a concurrent derivatization-DLLME extraction, and (2) the combination with a relatively inexpensive determination technique, as GC-MS, for the sensitive and selective determination of trace levels of benzotriazolic compounds in complex aqueous matrices. The performance of the developed method, particularly the achieved limits of quantification (LOQs) and the possibility to individually quantify isomeric benzotriazoles, is compared to that corresponding to previously published approaches, most of them using more sophisticated determination techniques.

7.2 EXPERIMENTAL

7.2.1 STANDARD, SOLVENT AND MATERIAL

Standards of BTri (98%), 4-methyl-1H-benzotriazole (4-TTri; 100%), 5-methyl-1H-benzotriazole (5-TTri; 98%), 5,6-dimethyl-1H-benzotriazole (XTri; 99%) and 1H-benzotriazole-(ring-d_4) solution (BTri-d_4), 10 μg mL^{-1} in acetone used as internal surrogate (IS) through derivatization and liquid microextraction steps, were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Two different standards of 5-chloro-1H-benzotriazole, with nominal purities of 98% and 99% were acquired from TCI (Zwijndrecht, Belgium) and Sigma-Aldrich, respectively. Stock solutions of the above compounds and diluted mixtures, used to spike water samples employed during optimization of extraction conditions, were prepared in acetonitrile and stored at 4ºC for a maximum of 2 weeks. A standard of 1-acetyl-1H-benzotriazole (97%) was also provided by Sigma-Aldrich.

Methanol and acetonitrile (HPLC-grade) were from Merck (Darmstadt, Germany). Acetone, toluene, chlorobenzene, carbon tetrachloride and 1,1,1-trichloroethane (trace analysis grade) were provided by Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Sodium acetate, acetic acid, sodium bicarbonate ($NaHCO_3$), disodium hydrogen phosphate (Na_2HPO_4) and acetic anhydride were also obtained from Sigma-Aldrich. Cellulose acetate membrane filters (0.45 μm pore size) were purchased from Millipore (Bedford, MA, USA).

Acetylated derivatives of target compounds, used during optimization of GC-MS determination conditions, were prepared as described elsewhere [157]. In brief, 10 mL of ultrapure water, containing a 0.8% (w/v) of Na_2HPO_4, were spiked with benzotriazole standards prepared in acetonitrile. Thereafter, 150 μL of acetic anhydride were poured into the same vessel, 5 mL of toluene were added and vials were manually shaken for 2 min. Derivatized species were concentrated in the upper organic phase (toluene), which was recovered using a Pasteur pipette before GC-MS analysis. In the particular case of BTri, the commercially available acetylated standard was also used.

7.2.2 SAMPLES AND SAMPLE PREPARATION

Grab samples of treated wastewater were obtained from different STPs located in Galicia (Northwest Spain); moreover, time-proportional 24-hour composite samples were received from the inlet stream of a STP serving a 100000 inhabitants city, in the same region. River water was obtained from two pristine creeks and the river receiving the discharge of the above STP.

Optimization of acetylation and DLLME conditions was performed with spiked (0.050 to 20 ng mL^{-1}) aliquots of ultrapure water, adjusted at different pHs, considering also different volumes of derivatization reagent (acetic anhydride) dispersant and extractant solvents. Extractions were performed in conical bottom glass tubes (nominal volume 12 mL), which were manually shaken during derivatization and microextraction steps. Thereafter, tubes were centrifuged and the settled drop of extractant (case of chlorinated solvents) recovered after removal of the upper aqueous phase. When using toluene as extraction solvent, the floating organic phase, together with some water, was transferred to a conical insert (0.3 mL volume)

to improve phase separation, recovering enough volume of toluene to be handled with the autosampler of the GC-MS instrument. Sample preparation conditions were optimized following uni- and multi-variate strategies based on the use of experimental factorial designs. In the latter case, the Statgraphics software (Statpoint Technologies, Warrenton, VA, USA) was used for experimental design creation and analysis.

Under optimal conditions, samples (10 mL) were first mixed with 1 mL of Na_2HPO_4 (8%, w/v) in the DLLME tube. Acetylation and microextraction of target compounds were simultaneously carried out by addition of a ternary mixture, consisting of 100 μL of acetic anhydride, 1.5 mL of acetonitrile and 60 μL of toluene. Reaction and centrifugation (3000 rpm) times were set at 1 and 5 min, respectively. After phase separation, as described above, around 30 μL of toluene could be recovered for GC-MS analysis.

7.2.3 GC-MS CONDITION

Acetylated compounds were determined by GC-MS. The gas chromatograph was an Agilent (Wilmington, DE, USA) 7890A model, equipped with a split/splitless injector and connected to a quadrupole MS spectrometer (Agilent MSD5975C), which was furnished with an electron impact (EI) ionization source. Compounds were separated with an Agilent HP-5MS capillary column (30 m x 0.25 mm i.d., d_f: 0.25 μm) using helium (99.999%) as carried gas, at a constant flow of 1.2 mL min^{-1}. The GC oven was programmed as follows: 80 ºC (held for 2 min), rate at 10 ºC min^{-1} to 280 ºC (held for 6 min). Injections (2 μL) were done in the splitless mode, with the solenoid valve switching to the split mode after 1 min. EI source, quadrupole and transfer line temperatures were maintained at 230 ºC, 150 ºC and 280 ºC, respectively. GC-MS chromatograms were recorded in the SIM mode, selecting two different ions per compound, Table 7.1.

Table 7.1: Abbreviations, retention times and GC-MS recorded ions for acetylated species

Compound	Abbreviation	Retention time (min)	Molecular Weight	Quantification (qualifier) ions, m/z values
1H-benzotriazole	BTri	9.76	119.1	133 (161)
4-methyl-1H-benzotriazole	4-TTri	10.99	133.2	104 (175)
5-methyl-1H-benzotriazole	5-TTri	11.31	133.2	104 (175)
5-chloro-1H-benzotriazole (2 isomers)	ClBTri1 ClBTri2	11.71 11.83	153.6	195 (197)
5,6-dimethyl-1H-benzotriazole	XTri	13.16	147.2	118 (189)
1H-Benzotriazole-(ring-d4) (I.S.)	BTri-d_4	9.74	123.2	137 (165)

Identities of acetylated benzotriazoles, and particularly the existence of two isomers in commercial 5-chloro-1H-benzotriazole standards, were confirmed using a second GC-MS system, equipped with an hybrid quadrupole time-of-flight (QTOF), 7200 model from Agilent, mass analyzer. Chromatographic conditions, EI source and transfer line temperatures were set to same values as those used in the single quadrupole GC-MS system. Moreover, an equivalent capillary column was installed in the GC-QTOF-MS system. Accurate MS spectra were recorded in the m/z range from 50 to 500 units with the spectrometer operated in the 2 GHz mode (full-width half-maximum mass resolution 5000 at m/z 131).

7.2.4 DLLME PERFORMANCE AND SAMPLES QUANTIFICATION

The efficiency of the sample preparation process, under optimized conditions, was evaluated using enrichment factors (EFs). They were defined as the ratio between the concentration of each compound in toluene extracts and those added to the water sample [166,167]. The concentration of BTri in the former solution was determined against a calibration curve built with a commercial standard of this acetylated compound. Acetylated derivatives of the rest of benzotriazoles, at different concentrations, were prepared as reported in section 7.2.1. Given

that the exact volume of the floating toluene phase is hard to establish, the absolute extraction efficiencies of the DLLME method were not calculated.

Potential variations of extraction efficiencies among ultrapure, surface and wastewater samples were evaluated using relative recoveries (%R) defined as follows:

$\%R = [(A_s-A_b)/A_r] \times 100$.

A_s is the response (analyte/IS peak areas) measured in the extract from a spiked sample, A_b is the response of the extract from a non-spiked fraction of the same sample, and A_r is the response measured in the extract from an aliquot of ultrapure water spiked at the same concentration level. The calculated %R values remained around 100%, a fact that indicates small variations in the efficiency of the acetylation-DLLME process for different matrices. Therefore, concentrations of benzotriazoles in environmental water samples were established by comparison with aliquots of ultrapure water, spiked with target species at different concentration levels (from 0.05 to 20 ng mL^{-1}) and containing the same level of IS (1 ng mL^{-1}).

7.3 RESULTS AND DISCUSSION

7.3.1 PRELIMINARY EXPERIMENTS

Pervova and co-workers [157] reported, for the first time, the acetylation of BTri with the aim of improving the performance of its GC-MS determination. Thereafter, the same procedure was applied to 4-TTri and 5-TTri [169]. In both cases, acetylation was performed in aqueous media, in presence of a basic catalyzer and analytes were further extracted, by conventional LLE, with toluene. This strategy was extrapolated to the rest of compounds involved in this study, introducing some changes regarding the type of base and volumes of acetic anhydride and toluene. Whatever the tested derivatization parameters, under chromatographic conditions reported in the experimental section, all compounds rendered a single, well-defined peak corresponding to the acetylated derivative, whose identity was verified on the basis of low and high resolution MS scan spectra and, in case of BTri, by injection of a commercially available

acetylated standard. However, in the case of 5-chloro-1H-benzotriazole (nominal purity above 98%), two peaks with the same MS spectra and similar intensities were observed.

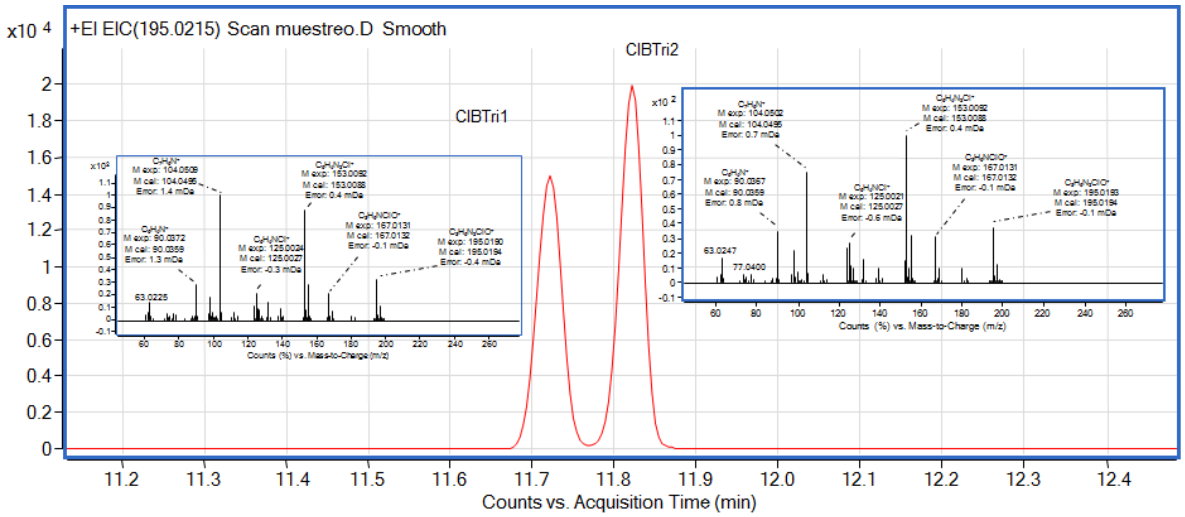

Figure 7.1: extracted ion chromatogram after acetylation of 5-chloro-1H-benzotriazole

Figure 7.1 shows the extracted ion chromatogram (extraction window 50 ppm) and accurate MS spectra, acquired with the GC-QTOF-MS system, after acetylation of 5-chloro-1H-benzotriazole. The EI-MS spectra were identical for both peaks; thus, it was assumed that commercial standards of 5-chloro-1H-benzotriazole correspond in fact to a mixture of 4-chloro and 5-chloro isomers. Although in previous published LC-MS methods, the existence of one single peak for 5-chloro-1H-benzotriazole has been reported [153, 168], the increased resolution provided by the GC capillary column for acetylated derivatives allowed the separation of both isomers. Quantification and identification ions used during this work for acetylated derivatives of target compounds, and the IS, together with the corresponding retention times are summarized in Table 7.1. In case of chloro-1H-benzotriazole, isomers were labeled as ClBri1 and ClBri2, assuming that the commercial standard is a 1:1 mixture of both species.

7.3.2 OPTIMIZATION OF SAMPLE PREPARATION CONDITIONS

Derivatization conditions and DLLME setup

Performance of acetylation reactions in aqueous solution can be affected by the type of basic catalyzer and the pH of the solution; moreover, when combined with DLLME, CO_2 bubbles might disturb separation of aqueous and extractant phases. Fractions (10 mL) of a spiked (3 ng mL^{-1}) ultrapure water sample were mixed with 1 mL of two different bases ($NaHCO_3$, pH 8; Na_2HPO_4, pH 9; both 5% w:v). Additional experiments were also performed using ultrapure water (pH 6), without any catalyzer, and samples adjusted at pH 5 with 1 mL of a sodium acetate-acetic acid (1 M) buffer. Then, 0.150 mL of acetic anhydride were added and the mixture was shaken for 2 min. A binary extraction mixture, consisting of 1 mL of acetone and 0.1 mL of chlorobenzene, was used for DLLME in all cases. Figure 7.2 shows the normalized responses (peak areas) obtained for acetylated compounds under above conditions.

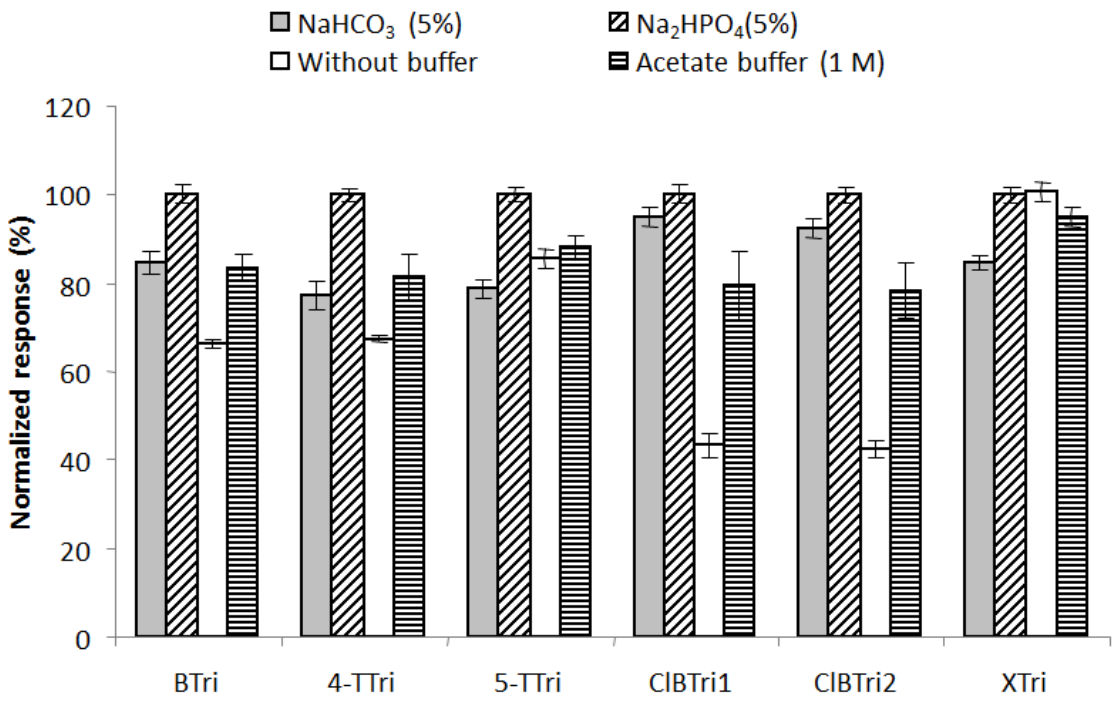

Figure 7.2: normalized responses (peak areas) obtained for acetylated compounds under different conditions

The highest responses were achieved using Na_2HPO_4, which was selected as catalyzer of the acetylation reaction in further experiments.

The effects of acetic anhydride volume (50-150 μL), derivatization time (2-6 min) and Na_2HPO_4 concentration (2-8%) were investigated using a Box-Behnken experimental design, with 3 central points and a total of 15 experiments. DLLME extractions were performed under conditions reported in the above section, with ultrapure water samples spiked again at 3 ng mL^{-1}. Responses for each compound in these experiments were analyzed by the Statgraphics software to obtain the main effects and two-factor interactions corresponding to variables involved in the design. Table 2 compiles the standardized main effects.

Table 7.2. Standardized main effect values for variables involved in the Box-Behnken experimental design

Compound	Acetic anhydride volume (50, 100 and 150 μL)	Reaction time (2, 4 and 6 min)	Na_2HPO_4 concentration (2, 5 and 8%)
BTri	0.87	-3.05[a]	6.28[a]
4-TTri	0.88	-2.11	5.81[a]
5-TTri	1.75	-1.24	3.02[a]
ClBTri1	0.85	-1.69	3.38[a]
ClBTri2	0.61	-1.63	3.75[a]
XTri	2.01	-0.81	1.42

[a] Significant effects at the 95% confidence level

The sign of main effects, positive or negative, corresponds to an improvement or a decrease in the acetylation step efficiency, respectively; whereas, the absolute values are correlated to the variation in the response of a given analyte when the associated variable moves from the low to the high level, within the domain of the design. The statistical significance boundary was established at the 95% confidence level.

The Na_2HPO_4 concentration was the most relevant variable, with a positive and statistically significant influence on the acetylation process for 5 of the 6 compounds. For XTri presented

a positive influence although it did not reach the statistical significance level. The reaction time followed an opposite trend, showing a negative effect on the yield of the derivatization, being just statistically significant for BTri. Finally, the acetic anhydride volume, despite exerting a positive influence on the process, remained non-significant. Based on above results, the phosphate buffer concentration was set at the highest level (8%) and the volume of acetic anhydride fixed in the intermediate value (100 µL). The negative, although in most cases non-significant, effect of the derivatization time in the responses of acetylated species suggests that (1) acetylation of benzotriazoles is a fast process and that (2) derivatives might be slowly hydrolyzed to the free forms in contact with aqueous sample at basic pH. Taking into account these considerations, the possibility of combining acetylation and DLLME processes in the same step, as reported in case of chlorophenol compounds [170], was further evaluated.

To this end, we compared the responses obtained under above conditions, considering an acetylation time of 2 min, followed by DLLME extraction (two-step approach) and adding the acetic anhydride (100 µL) to the binary mixture of acetone (1 mL) and chlorobenzene (100 µL) (single-step procedure). In both cases, manual shaking and centrifugation (3000 rpm) times were 2 and 5 min, respectively. Figure 7.3 shows the obtained normalized responses for each compound.

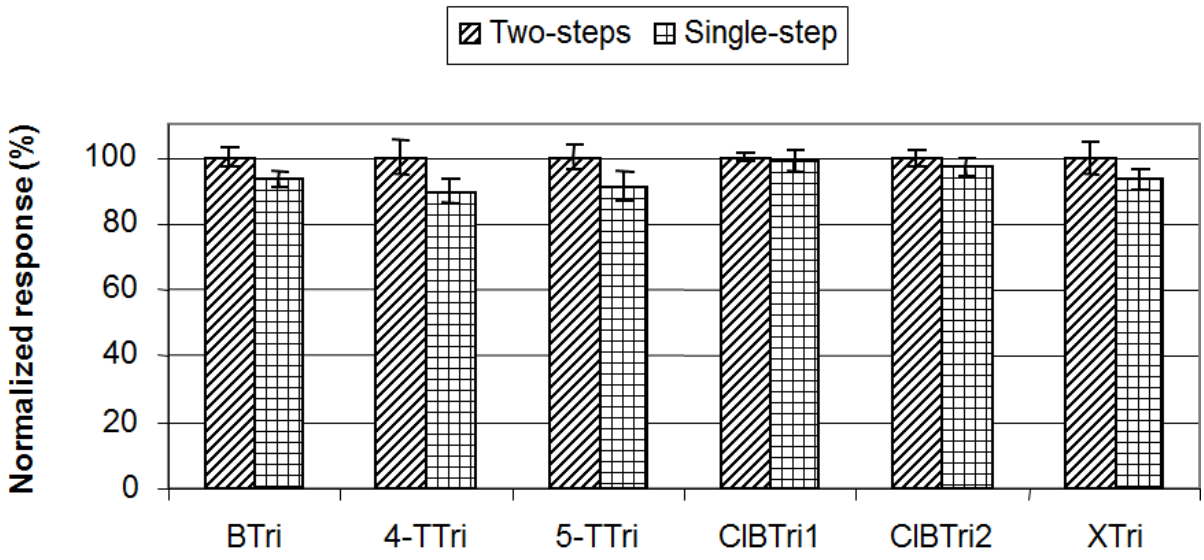

Figure 7.3: comparison between two-step approach and single-step procedure

No significant differences between the results provided by the two methodologies are observed for ClBTri1, ClBTri2 and XTri. For the other 3 benzotriazoles, responses for the single step approach represented between 90 and 95% of those attained in two steps. On the view of these results, in order to save time and to reduce sample manipulation, in further experiments analytes acetylation and concentration were simultaneously performed.

DLLME conditions

Selection of a suitable extraction solvent is one of the most important issues during method development in DLLME. Three solvents with higher density than water, commonly used in DLLME [163] (chlorobenzene, carbon tetrachloride and trichloroethane), and toluene, as a lighter than water alternative, were compared on the basis of their affinity for acetylated benzotriazoles. In all cases, the volume of extractant was 100 µL.

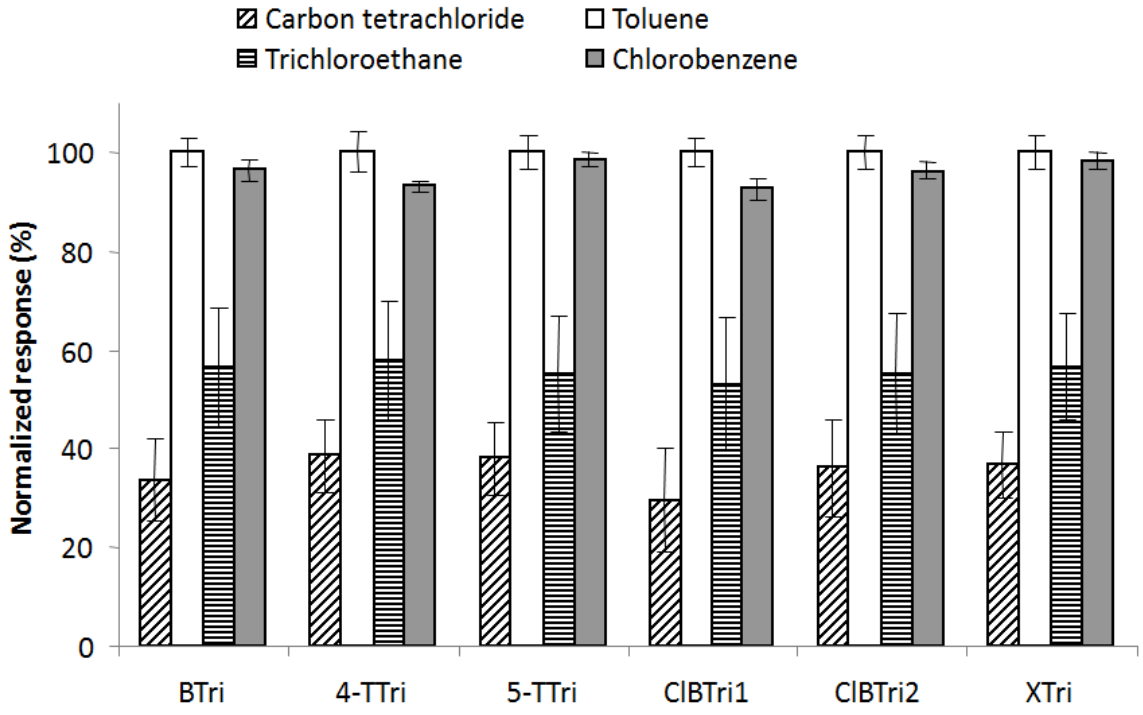

Figure 7.4: normalized peak areas for each compound as function of the type of extractant

Carbon tetrachloride and trichloroethane provided the lowest responses and the highest variabilities for all species, whereas similar peak areas were measured for toluene and

chlorobenzene. Likely, π-π interactions established between acetylated benzotriazoles and both aromatic solvents are responsible for their higher extraction efficiencies versus chlorinated alkanes. Despite separation of the floating toluene extract was more complex than direct collection of the settled phase of chlorobenzene, the former solvent was preferred as extractant because of its lower toxicity. As reported in the experimental section, firstly, the upper phase of the extraction tube was transferred to a narrow (i.d. 3 mm) conical insert, where a neat interface between toluene and the aqueous phase was obtained.

The type of dispersant (methanol, acetone and acetonitrile) exerted a minor effect in the responses of derivatized compounds (data not shown); however, acetone led to a peak with a retention time close to that of 5-TTri and same nominal m/z values and methanol showed the highest variability. Therefore, acetonitrile was selected as dispersant.

Figure 7.5 compares the peak areas obtained combining two different volumes of toluene (60 and 120 µL) with four of acetonitrile (0.5, 1.0, 1.5 and 2 mL).

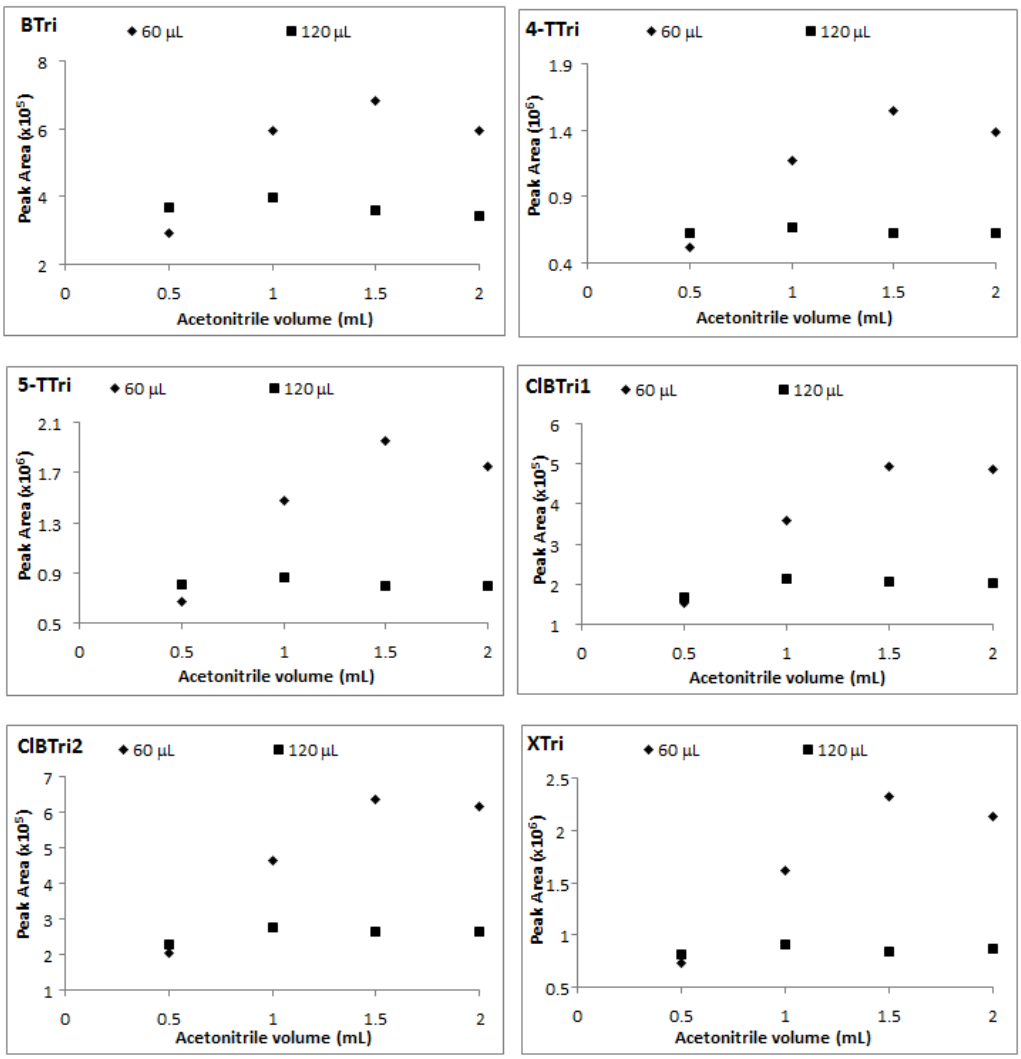

Figure 7.5: peak area in function of extractant and dispersant volume

In all cases, 100 µL of acetic anhydride were incorporated, as derivatization reagent, in the ternary extraction mixture. With the only exception of the lowest dispersant volume, higher responses were achieved using 60 µL of toluene than with 120 µL. For the former extractant volume, the increase in the responses of the analytes with the volume of acetonitrile can be explained since a more efficient dispersion of toluene droplets in the aqueous sample is achieved. At 2 mL of acetonitrile, the increased solubility of acetylated analytes in the aqueous phase led to a small reduction in the efficiency of their extraction. Thus, 60 µL and 1.5 mL were adopted as toluene and acetonitrile optimal volumes. Under these conditions, 25-30 µL of toluene could be recovered at the end of phase separation process.

The influence of the ionic strength on the efficiency of the DLLME was evaluated comparing the responses obtained without and with addition of 1 g of NaCl to water samples. No significant variations were noticed in the responses measured for acetylated compounds; thus, no salt was used in further extractions. The extraction time, after addition of the ternary acetylation-extraction mixture, was varied between 1 and 5 min, whereas centrifugation (3000 rpm) times of 5, 10 and 15 min were tested. None of these factors modified the performance of the extraction; thus, extraction and centrifugation steps were limited to 1 and 5 min, respectively.

7.3.3 PERFORMANCE OF THE METHOD

Linearity of the proposed methodology was investigated with ultrapure water aliquots fortified with increasing concentrations of target benzotriazoles (from 0.050 to 20 ng mL^{-1}, n=7 levels), maintaining the IS at 1ng mL^{-1}. The corrected responses (peak area/IS peak area) for each compound were plotted against their concentrations in the water samples and fitted to a linear model. Determination coefficients (R^2) values for the obtained graphs varied from 0.995 up to 0.9997. Regarding reproducibility, nine extractions were carried out in three different days with samples spiked at two concentration levels, 0.2 ng mL^{-1} and 2 ng mL^{-1}. Relative standard deviation (RSDs, %) of corrected responses remained between 2 and 10%. Efficiency of the proposed method was evaluated with EFs, calculated as defined in the experimental section, for a sample spiked at the 10 ng mL^{-1} level. Analytes were concentrated between 93 times (BTri) and 172 times (XTri), Table 7.3.

Table 7.3: Linearity, enrichment factors (EFs), inter-day precision and limits of quantification (LOQs) of the method

Compound	Linearity (R^2, 0.050-20 ng mL^{-1})	EFs	Reproducibility (RSDs,%) (n=9 replicates, 3 days)		LOQs (ng mL^{-1})
			[a]0.2 ng mL^{-1}	[a]2 ng mL^{-1}	
BTri	0.9997	93 ± 5	2	2	0.045
4-TTri	0.9995	134 ± 7	4	5	0.007
5-TTri	0.9991	134 ± 7	6	4	0.009
ClBTri1	0.995	161 ± 10	8	10	0.080
ClBTri2	0.998	171 ± 7	9	7	0.060
XTri	0.9993	172 ± 9	7	8	0.013

[a]Addition level

The limits of quantification (LOQs) of the method were calculated as the concentration of each compound providing a response 10 times higher than the baseline noise at the retention time of each compound in procedural blanks. BTri was noticed at low levels in procedural blanks, therefore, its LOQ was calculated as 10 times the standard deviation value for this peak in five consecutive procedural blanks divided by the slope of the calibration curve. The attained LOQs varied between 0.007 ng mL^{-1} for 4-TTri and 0.08 ng mL^{-1} for ClBTri1, Table 7.3. Regarding other microextraction applications, these values are significantly lower than those obtained by DLLME, using tri-n-butylphosphate as extractant, and LC determination (0.1-7.3 ng mL^{-1}) [168] and in the same order than those reported using polyethersulfone solid-phase microextraction and LC-QTOF MS (0.005-0.1 ng mL^{-1}) [153], with the advantage of employing a much faster sample preparation approach. LOQs summarized in Table 7.3 are also equivalent to those obtained by SPE combined with LC-MS/MS [140, 147-150, 160-162], LC-LTQ FT Orbitrap MS [151] and GC x GC-TOF-MS [156, 158] requiring a less sophisticated instrumentation.

Potential changes in the performance of the sample preparation procedure among water samples with different complexities was investigated comparing the responses obtained for ultrapure and different water samples spiked at two different concentration levels (0.5 ng mL^{-1}

and 10 ng mL^{-1}). Obviously, non-spiked aliquots of environmental water samples were also prepared. The relative recoveries values, calculated as described in the experimental section, varied between 91 ± 11% and 116 ± 3%, Table 7.4. Therefore, after IS correction, comparison of responses measured for environmental water samples with those attained for spiked aliquots of ultrapure water can be used as quantification approach.

Table 7.4: Relative recoveries for samples spiked at two different concentrations levels (0.5 ng mL^{-1} and 10 ng mL^{-1}), n=4 replicates

Compound	Tap water (0.5 ng mL^{-1})	River water (0.5 ng mL^{-1})	Effluent (10 ng mL^{-1})	Influent (10 ng mL^{-1})
BTri	103 ± 14	98 ± 6	108 ± 2	111 ± 2
4-TTri	107 ± 16	101 ± 4	109 ± 4	109 ± 4
5-TTri	106 ± 15	101 ± 2	109 ± 2	109 ± 2
ClBTri1	91 ± 11	99 ± 7	107 ± 3	107 ± 3
ClBTri2	104 ± 9	116 ± 3	106 ± 2	106 ± 2
XTri	97 ± 2	108 ± 4	108 ± 3	108 ± 3

7.3.4 REAL SAMPLE ANALYSIS

Table 7.5 reflects BTri, 4-TTri and 5-TTri levels in 24-h composite raw wastewater samples obtained, during a week, from the same STP serving a 100.000 inhabitants population.

Table 7.5: Concentrations (ng mL^{-1}) of BTri and tolyltriazoles in 24-h composite raw wastewater, and masses (g day^{-1}) entering an urban STP during a seven days sampling campaign, n=3 replicates

Day	Concentration (ng mL^{-1}) ± SD			Ratio 5-/4-TTri	Water volume (m^3 day^{-1})	Mass (g day^{-1})		
	BTri	4-TTri	5-TTri			BTri	4-TTri	5-TTri
1	1.94 ± 0.08	0.47 ± 0.02	0.56 ± 0.04	1.2	58410	113	27	33
2	1.31 ± 0.02	0.32 ± 0.01	0.37 ± 0.01	1.2	58909	77	19	22
3	1.35 ± 0.02	0.24 ± 0.01	0.30 ± 0.01	1.3	62813	85	15	19
4	1.43 ± 0.04	0.35 ± 0.01	0.42 ± 0.01	1.2	61505	88	22	26
5	0.62 ± 0.01	0.32 ± 0.03	0.27 ± 0.01	0.8	58024	36	19	16
6	0.46 ± 0.02	0.20 ± 0.01	0.21 ± 0.01	1.1	66050	30	13	14
7	0.66 ± 0.02	0.17 ± 0.01	0.18 ± 0.01	1.1	70394	46	12	13
Average	1.11	0.30	0.33	1.1	62301	69	19	21

The rest of compounds remained under their LOQs; although, ClBTri isomers were detected in some samples. The average raw wastewater concentration of BTri (1.11 ng mL^{-1}) was significantly lower than that found in German STPs influents (12 ng mL^{-1}) [140,141] and other Spanish locations (7.3 ng mL^{-1}) [158]. Average individual concentrations of tolyltriazoles represented around 25% of that corresponding to BTri. The ratios of their concentrations (5-TTri/4-TTri) varied from 0.8 to 1.3, with an average value of 1.1, which is in concordance with previous studies. While Weiss et al [140] reported a 5-TTri/4-TTri ratio of 1.06, Casado et al [153] found values between 0.84 and 1.04. Taking into account the daily processed water volume (c.a. 62000 m^3), the global mass discharge of the above corrosion

inhibitors in the plant was estimated. The average daily input of BTri was 69 g, followed by 20 g of 4- and 5-TTri. Thus, the STP receives a total of 0.11 kg day^{-1} of benzotriazoles, which is in a relatively low amount when compared with 9.72 kg day^{-1} recently reported for a STP processing a 12-times higher input of wastewater [150].

Table 7.6. Concentrations (ng mL^{-1}) in grab samples of river and treated wastewater, n=3 replicates

Code	Type	Concentration (ng mL^{-1}) ± SD			Ratio
		BTri	4-TTri	5-TTri	5-/4-TTri
1	River	0.025 ± 0.003	n.d.	n.d.	--
2	River	0.051 ± 0.003	0.016 ± 0.001	0.009 ± 0.002	0.6
3	River	0.144 ± 0.005	0.102 ± 0.003	0.102 ± 0.005	1.0
4	Sewage	0.64 ± 0.01	0.37 ± 0.01	0.39 ± 0.01	1.1
5	Sewage	0.27 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.9
6	Sewage	0.19 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	1.0
7	Sewage	0.68 ± 0.02	0.26 ± 0.02	0.25 ± 0.02	1.0
8	Sewage	0.41 ± 0.01	0.21 ± 0.02	0.20 ± 0.03	1.0
9	Sewage	0.15 ± 0.01	0.19 ± 0.01	0.090 ± 0.004	0.5

Table 7.6 compiles the concentrations of BTri, 4-TTri and 5-TTri (rest of compounds remained undetected) in grab samples of river water (codes 1-3) and the outlet streams (codes 4-9) of different STPs. River water samples codes 1 and 2 were collected from relatively pristine creeks, whereas sample number 3 was taken 5-km downstream the discharge of a STP. As regards treated wastewater samples, BTri usually remained at higher levels than tolyltriazoles; however, differences between their concentrations were lower than those found for raw wastewater samples compiled in Table 7.5. Finally, 5-TTri/4-TTri ratios in treated wastewater again remained around the unit (Table 7.6), except for sample code 9. This sample corresponds to the only STP applying UV disinfection after the secondary (activated sludge) treatment tank.

7.4 CONCLUSION

A simple, rapid, and low cost methodology has been developed for the determination of several benzotriazolic derivatives in different aqueous matrices. The protocol requires a very low volume of sample and just a few microlitres of organic solvent for the microextraction. It enables the concurrent acetylation and microextraction processes with sample preparation requiring just 10 minutes. GC-MS, a relative accessible instrumentation, reaches LOQs comparable to those reported using more sophisticated systems such as LC-MS/MS or GC x GC-TOF-MS. Moreover, the resolution between 4-TTri and 5-TTri, and also between ClBTri isomers, is improved compared to that provided by LC-MS based methods. In summary, the described procedure constitutes an appealing alternative to monitor the levels and the behavior of several benzotriazoles during wastewater treatments and also to investigate their fate in the aquatic environment.

Further studies will be performed on foods cultivated using waters of the river in which benzotriazoles were found. In doing so, it will be possible to study the possible contamination of the foods due to the use of irrigation waters polluted by benzotriazoles.

CHAPTER 8

OVERALL CONCLUSION

In conclusion, new methods for analysis of foods were developed, based on the fruitful coupling of different instrumental profiling methods to chemometric data processing techniques, which allow reliable quality control and traceability of the origin of the product. In this respect, on one hand, chromatographic fingerprinting of the phenolic fraction proved to be a valid secondary traceability indicator for oil and honey samples.

On the other hand, thanks to the many benefits provided by NIR spectroscopy coupled with chemometric techniques, it was possible to build models of classification and regression which allowed to discriminate different samples, providing an additional tool to combat fraud. In this framework, particular attention was posed to the respect of the principles of "Green Chemistry", which has now become the focus of the chemistry of the new millennium. Indeed, the use of NIR spectroscopy allowed developing methods with less impact on the environment, humans and higher performance compared to existing methods for analysis of foods. But it is necessary to point out that, in all the examined cases, a key role is played by chemometrics. Indeed, the possibility of using a not selective fingerprinting technique such as NIR for calibration and classification, without needing any separation step or sample pretreatment is only made possible by the use of chemometric data processing which allow to mathematically manage the presence of interferents and other sources of unwanted variability in the signals. Furthermore, chemometric proved to be essential also for all the other studies presented in this PhD research, and ubiquitous in all the stages of the analytical process, starting from sampling strategies and experimental design to the final validation of the results obtained.

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